EFFECT OF HZE RADIATION AND DIETS RICH IN FIBER AND n-3 POLY UNSATURATED FATTY ACIDS (n-3 PUFA) ON COLON CANCER IN RATS

A Thesis

by

ANNA ANATOLIEVNA GLAGOLENKO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2005

Major Subject: Health Physics

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Approved as to style and content by:

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May 2005

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ABSTRACT

Effect of HZE Radiation and Diets Rich in Fiber and n-3 Poly Unsaturated Fatty Acids (n-3 PUFA) on Colon Cancer in Rats. (May 2005)

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This study examines the carcinogenic effect of HZE radiation and protective effects of different types of diets against colon carcinogenesis in a rat model.

The effect of HZE radiation on health state and colon cancer development was evaluated. HZE radiation was found to suppress food consumption ($P<0.0001$) leading to lower body weight gain of irradiated rats when compared to the non-irradiated rats ($P<0.05$). The animals exposed to HZE radiation were found to start dying and/or getting pathologies 11 weeks earlier and at the end of the study had morbidity/mortality rate 14.2% higher ($P=0.0005$) than non-irradiated rats. There was no significant effect of HZE radiation on colon cancer incidence.

The effects of dietary fibers and oils on health state and colon carcinogenesis were evaluated. Morbidity/mortality was found to be delayed in rats fed with pectin-based diets when compared to cellulose-based diet, regardless of radiation treatment. Similarly, fish oil was found to beneficially affect health of the experimental animals when compared to corn oil. Ten- and twenty-week delayed morbidity/mortality for irradiated and non-irradiated groups, respectively, was observed for rats fed with fish

oil-based diets when compared to corn oil-based diets. Fish oil was also found to significantly reduce colon tumor incidence and multiplicity in non-irradiated rats ($P<0.05$). A similar trend was observed for the irradiated animals. No significant effect of fiber on colon cancer incidence was found.

Finally, the effect of diets on general health and colon cancer development was investigated. Rats fed with corn oil/cellulose diet started dying and/or getting a disease earlier than rats fed with other diets, regardless of radiation treatment. The effect of diet on colon cancer development was found to depend on radiation treatment. Thus, in the absence of radiation treatment fish oil/cellulose was found to significantly reduce tumor incidence and multiplicity when compared to corn oil/pectin diet ($P<0.05$). In the presence of radiation treatment fish oil/pectin was found to lower the values of tumor incidence and tumor multiplicity, though the data obtained were not significant.

This thesis is dedicated to my uncle, Vladimir Ivanovich Borisov, who I loved very much. I hope he is in a better place now.

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I would like to express my heartfelt thanks to my co-chairs Dr. John Ford and Dr. Joanne Lupton for introducing me to the astonishing world of Health Physics and Nutrition Sciences, for the enthusiasm and guidance they provided throughout this project, and for the kindness and encouragement they gave me when I needed them most. I would also like to thank the members of my advisory committee: Dr. Leslie Braby and Dr. John Poston, for the time they invested in this work.

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My deepest gratitude to the people I was lucky to work with: my beloved Mrs. Stella Taddeo, my friend and amazing scientist Dr. Jairam Vanamala, beautiful and smart women Dr. Mee Young Hong and Lisa Sanders, and also Dr. Laurie Davidson, Evelyn, Nataša, Kim, Kristy, Anne, Tety, Cindy, John, Lori Lynn, Chris, Heather, Alicia, Caitlin, Lauren, Katee and Rebecca for their friendship, support and help during this study.

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CHAPTER I

INTRODUCTION

Constantly expanding human exploration in space is associated with various risk factors among which radiation exposure is considered to be of major importance. Studies have shown that airline pilots have an elevated occurrence of malignant neoplasms, such as melanoma (1), myeloid leukaemia (2), brain cancer and prostate cancer (3) which are inferred to be due to increased exposure to cosmic radiation. Radiation from galactic cosmic rays (GCR) is the predominant type of space radiation (4). Heavy charged particles (HZE) represent about one percent of total GCR radiation (5). Despite being a tiny fraction of the total incident particles, they contribute significantly to detrimental biological effects on members of a space crew (6, 7) due to their high linear energy transfer (LET). The detrimental effect of heavy ions has been described for different organs, including brain, skin and eyes (8-10). The National Research Council determined that the carcinogenic risk following irradiation by HZE particles is a high priority research area (11).

Data from more than 50 years of investigations including epidemiologic, case control and laboratory animal studies show clear evidence of carcinogenic effects of different types and doses of radiation on colon tissue. High colon cancer incidence has been detected for Japanese atomic bomb survivors (12) and patients treated with therapeutic irradiation of the pelvic region for various benign and malignant conditions

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This thesis follows the style and format of *Cancer Research*.

(13, 14). Cancer was found to be a leading cause of death among radiologists, with colon cancer being one of the most frequent types of cancer (15). Multiple animal investigations have detected colon cancer induced by various doses of γ, x, β and neutron radiation (16-21). Colon cancer is the second leading cause of cancer-related death after lung/bronchus cancer in a United States population, with a median age at death of 75 (22). There is a higher incidence detected in black males when sex and ethnicity are examined. Although the incidence and mortality rates from colon cancer have decreased during the last ten years, the estimated numbers of new colon cancer cases and death for year 2004 still remain very high - 106,370 new colon cancer cases and 56,730 death cases estimated. The complex exposures to cancer-inducing agents, including HZE irradiation of a space crew, probably result in markedly increased risk of colon cancer. By identifying practical countermeasures to radiation-induced tumorigenesis we would be able to diminish the impact of radiation on the risks of space exploration.

1.1. BACKGROUND

1.1.1. HZE and colon cancer pathogenesis

It is well-documented that ionizing radiation induces production of reactive oxygen species (ROS) such as superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) (23, 24), which can result in permanent DNA damage via oxidation (25, 26). The three most common types of damage at the chromatin level include (in order of abundance) DNA base damage, single strand breaks (SSB) and double strand breaks (DSB) (27). While the first two types of damage are usually repaired readily (28, 29), fast and accurate DSB repair may fail. This may cause subsequent DNA rearrangements such as fragmentation, deletion and/or chromosome aberrations (30) resulting in loss of genetic information that may lead to neoplastic initiation or progression.

Genomic instability, a delayed effect associated with ionizing radiation and characterized by increased rate of genetic alterations in the progeny of irradiated cells many generations after the irradiation, also may lead to neoplastic changes (31, 32). Though the exact mechanism underlying genomic instability is yet to be established, it is supposed to be a result of complex prolonged processes involving ROS (33, 34) and such non-targeted radiation-related effects as bystander and death-inducing effects (35).

Together with particle energy, linear energy transfer (LET), which is a characteristic of the ionization density, affects the biological response to irradiation. HZE ions are characterized by high LET. Thus, while evident proof of DNA damage inflicted by various types of ionizing radiation has been reported in different *in vivo* and *in vitro* studies (36-43), the number of chromosome aberrations induced by HZE ions was found to be much higher than that from γ or x-rays. It has been shown for the harderian gland that the relative biological effectiveness (RBE) for charged particle carcinogenesis is LET dependent (44). The capacity of radiation to induce genomic instability was also reported to depend on LET (45).

It should be mentioned, that, at low doses, the mechanism of action of HZE radiation on subcellular structures is not yet fully investigated. Thus, it was recently found that low doses of Fe-ion radiation induce not only DSB (about 30% of all DNA damage reported) but also several classes of *clustered* DNA damage, which are defined as two or more lesions such as strand breaks, abasic sites or oxidized bases formed within few helical turns of the DNA (46). Their reparability and biological effect are not yet known. The latest data suggest that the damage inflicted on chromatin structures is directly proportional to the probability of the cell being hit by a single primary heavy ion and the dose response function has a linear relation (47, 48). This supports the theory that a no-threshold model can be applied to the dose-response relationship describing carcinogenic processes at low doses of irradiation by HZE.

1.1.2. Dietary fats and fibers as colon cancer preventive agents

Experiments carried out for over three decades have demonstrated that colon cancer risk is strongly related to the presence of such components as fats and fibers in a diet. It was found that diets rich in fibrous food are associated with reduced risk for colon cancer (49-51). In turn, high dietary fat consumption poses higher risks for colon cancer development in humans (52, 53) and in animals (54, 55).

Moreover, not only the amount of fat and fiber but also their composition is important. Thus, citrus fiber (highly fermentable) was shown to have no effect on 3,2'-dimethyl-4-aminobiphenyl (DMAB)-induced colon tumors in rats in contrast to wheat bran (poorly fermentable) (56), which was found to reduce both tumor incidence (number of animals with tumors) and tumor multiplicity (number of tumors per tumor bearing animal). In a similar study using another carcinogen, azoxymethane (AOM), the number of adenomas and adenocarcinomas of the colon was reduced in rats fed with wheat bran diet compared to those fed the control diet. In contrast, the number of adenomas but not the number of adenocarcinomas was reduced in rats fed the citrus pulp diet (57).

Oils rich in n-3 polyunsaturated fatty acids (n-3 PUFA), such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), have been shown to reduce colon cancer incidence compared to oils rich in n-6 polyunsaturated fatty acids such as linoleic acid (18:2n-6) (58-60). In particular, fish oil, high in n-3 PUFA, versus corn oil, rich in linoleic acid, is found to be protective against experimentally induced colon cancer in rats (61-65).

The combination of fish oil and pectin, which is a highly fermentable fiber, has been shown to have a synergistic protective effect on colon tumor incidence but the protective effect of these diet components has not been examined in relation to radiation-induced colon cancer (62).

1.1.3. Dietary fibers and colon cancer pathogenesis

Though multiple studies demonstrate the preventive nature of fibers against colon cancer, the underlying processes are not completely understood. Such factors as possible dilution and adsorption of carcinogens, co-carcinogens and/or promoters by dietary fiber may have a place (66), and possible reduction in transit time of luminal contents due to increased dietary fiber intake may result in less exposure of the gut lining to the above listed factors.

However, the main pathway of colon cancer suppression is related to the modulation of balance between colonocyte proliferation, differentiation and apoptosis. It is well known that colon cancer is a multi-stage process triggered by loss or mutation of multiple genes. Subsequent inactivation of tumor suppressor genes and/or mutations in oncogenes (67) leads to deregulation of cell proliferation or apoptosis. Studies found a reduction in apoptosis in colonic adenomas, carcinomas or colonic biopsies from colon cancer patients compared to healthy colon tissues (68-70). Abrasiveness of some types of fibers is thought to lead to increase in colonocyte proliferation (71). It has been reported that when proliferation/apoptosis balance is a subject of interest, the increased rate of apoptosis rather than decreased rate of proliferation suppresses colon carcinogenesis (62). Recent studies suggest that dependence of level of apoptosis on fiber fermentability is most probably the key explaining preventive role of fibers against colon cancer (56, 57, 72). Fermentability has a significant effect on intestinal microflora, such as anaerobic bacteria. Their metabolic products, short chain fatty acids (SCFAs), forming during fermentation of dietary fiber and resistant starch, have been recently shown in several studies to inhibit colon cancer (73, 74). However, the discrepancy between *in vivo* and *in vitro* studies to date (75) makes the role of SCFA in colon tumorigenesis a subject of debate.

1.1.4. Dietary fats and colon cancer pathogenesis

Multiple studies investigating effects of oils on colon tumorigenesis showed fish oil to be more protective than many other lipid sources (61, 62, 64, 65, 76). The mechanisms by which fish oil alters colon tumorigenesis are the subject of investigations and mostly consider its effect on colonocyte proliferation and apoptosis (62, 77-80). Among them, two major concepts are: inhibition of prostaglandin (PG) production (78) through decreased availability of arachidonic acid (AA) and reduced activity of cyclooxygenase-2 (COX-2, inducible enzyme for PGE_2 production) (81, 82, 76), and modulation of RAS gene mutation and expression (83-85) resulting in protein kinase C (PKC) triggered apoptosis (86-88).

Other studies also point to the fish oil-related decrease of the concentration of colonic secondary bile acids, namely deoxycholic and lithocholic acids (89), which were found to reduce colonic cell apoptosis (90). N-3 PUFA have also been reported to suppress angiogenesis (growth of new blood vessels from pre-existing endothelial cells) (91) via decrease of iNOS expression. A high level of angiogenesis is related to more malignant and invasive tumor pattern (92).

1.1.5. Justification of carcinogen agent

Currently 1,2-dimethylhydrazine (DMH) and its metabolites have found wide application in studies in animal models as targeted colonic carcinogens. Their mechanism of carcinogenesis is well-studied and represents multi-step molecular mutations in regulatory genes initiated by methylation of DNA bases by DMH derivatives (93). Azoxymethane (AOM), one of the DMH derivatives, which is characterized by improved stability in injection solutions, was extensively investigated in multiple experiments with rodents and found to have narrow carcinogenic effect targeted on the colonic epithelium.

1.1.6. Justification of rat model of colon cancer

At the present time, Sprague-Dawley rats, utilized in the current experiment, are one of the most widely used strains in carcinogenicity studies. They are currently being used as a model of Fe-ion-induced mammary cancer, in studies of brain signaling, behavioral responses and aging effects induced by exposure to Fe-ion radiation. Spontaneous tumors, though reported in Sprague-Dawley rats, had a very low incidence in the gastro-intestinal tract (less than 1% for small intestine and colon (94), and appeared in aged animals (94-97). Spontaneous tumors in male Sprague-Dawley rats that were the cause of death were found to be of non-intestinal origin and the mortality rate observed ranged from 35% to 51%. Male rats of Harlan Sprague-Dawley stock were reported to have lower spontaneous incidence of pituitary gland tumors, the main cause of death among the rats of this stock, and a survival rate about 65% (98). These indices are in compliance with U.S. Food and Drug Administration's Red Book regulating the length of a carcinogenicity study to 24 months maximum with at least a 50% survival at the end of study.

Finally, the AOM-induced colon cancer model has been extensively studied in rats and the carcinogenic processes involved in the development of colon cancer in rats were found to be comparable with those in humans (77).

CHAPTER II

MATERIALS AND METHODS

2.1. OVERALL EXPERIMENTAL DESIGN

The overall experimental design which is a 2x2x2x1 factorial design with two lipid treatments (fish oil versus corn oil), two fiber types (pectin and cellulose), two radiation exposures (1.0 Gy Fe-ion or no exposure) and carcinogen (azoxymethane) treatment is represented in Figure 1.

2.2. ANIMALS

All the rats were Sprague-Dawley male weanlings weighing 40-60 g on arrival. They were bred and maintained in the Harlan Teklad facility (Indianapolis, Indiana) and delivered to the Laboratory Animal Research Resource facility (LARR), Texas A&M University, College Station, Texas (non-irradiated rat groups) or Brookhaven National Laboratory (BNL), Upton, New York (irradiated rat groups) when 3 weeks old. They were housed in individual cages in a temperature 25^0C controlled room with a 12/12-hour light/dark cycle. The rats were provided with water and chow diet *ad libitum*. Following a five-day acclimatization period the animals were divided into four groups. Stratification by body weight was made so that the mean initial body weights of the groups did not differ. The chow diet was then shifted to the one of the four experimental diets for each group.

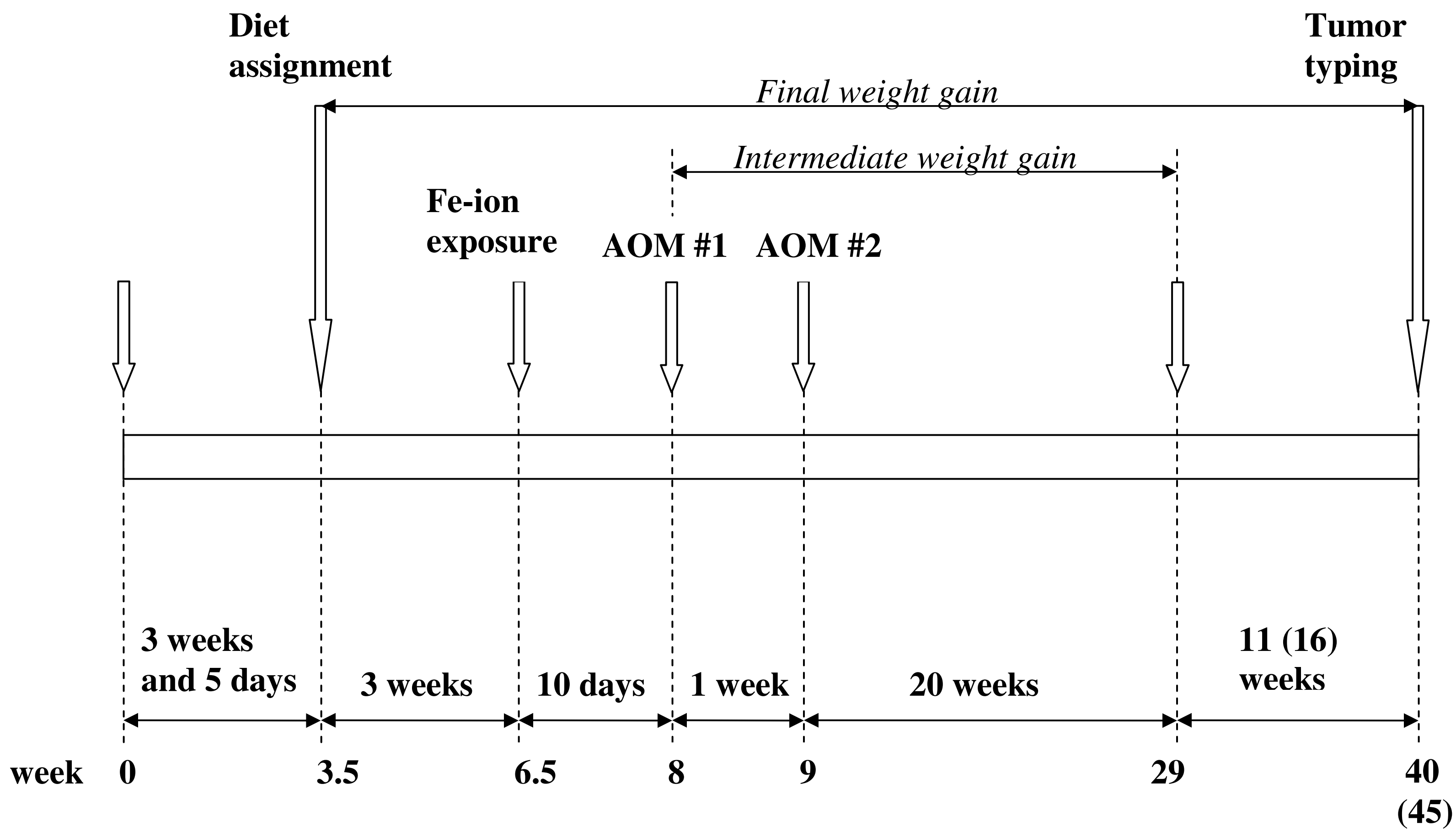


Fig. 1. Experimental design.

A total of 240 rats were used in the experiment. There were two groups of animals, with or without radiation exposure, with 120 rats in each group. Each of the two groups was further divided into two equal sets, of 60 animals each. Only one set of rats at a time was managed to ease maintenance and ensure quality care.

Three weeks after the rats were started on the experimental diets the rats belonging to the irradiated groups were exposed to a 1.0 Gy Fe-ion beam. The same day they were implanted with transponder chips programmed with information about rat diet, rat number and the day of carcinogen treatment. Irradiated rats were shipped from BNL to LARR, TAMU after three days of recovery. Upon arrival the rats were re-housed in individual cages.

A series of two azoxymethane (AOM) treatments were implemented 38 and 48 days after the rats started receiving experimental the diets (ten days and seventeen days after the radiation exposure for the irradiated groups). Three days after the second injection all the animals were transferred to the Kleberg Vivarium where all groups were housed in the same conditions until their euthanasia.

The first non-irradiated group of animals was killed 36 weeks after the second AOM injection. All remaining rats were killed 31 weeks after the second AOM injection. The change in termination schedule was caused by more rapid onset of morbidity with radiation exposure. In all cases euthanasia was accomplished by carbon dioxide asphyxiation followed by cervical dislocation.

While alive, the rats were carefully monitored for any signs of discomfort or sickness such as lack of food intake, weight loss, fecal blood/rectal bleeding, lethargy, aggressiveness or other changes in behavior. Each rat was manually examined daily. Rats with macroscopic tumors found at visual checkup or palpation were monitored and weighed daily from that point onward. Any animal expressing signs of sickness such as lethargy, rectal or outer tumor bleeding, continuous weight loss, continuous diarrhea, not eating, seizures or problems with locomotion were euthanized and the date recorded.

Food intake was determined 20 weeks after the second AOM injection for all animals and 36 weeks after the second AOM injection for the first non-irradiated group.

2.3. DIET

The 2x2 diet factorial design with two lipid treatments (fish oil versus corn oil) and two fiber types (pectin and cellulose) was used in the study. There were a total of 60 rats per diet treatment. The animals were assigned one of the four diets following five days of an acclimatization period starting upon arriving to BNL (irradiated groups) or LARR, TAMU (non-irradiated groups).

Harlan Teklad basal diet mixes (Harlan Teklad, Indianapolis, IN) and Degussa lipids (Degussa, Waukesha, WI) were used in all four experimental diets. A detailed diet composition is given in Table 1.

All diets were composed of a total of 6% fiber by weight according to the recommended level of 30 g fiber per day for humans and 15% (30% of energy) lipid by weight based on current recommendations for humans (US Public Health Service 1991). The major differences between the fatty acid compositions of the two lipid sources were the concentrations of eicosapentaenoic acid (EPA, 20:5n-3) (18.2%) and

Table 1 *Composition of the experimental diets*

	g/100 g
Dextrose, monohydrate	51.06
Casein	22.35
DL-Methionine	0.34
Mineral Mix, AIN-76A (170915) [1]	3.91
Vitamin Mix, AIN-76A (40077) [1]	1.12
Choline Bitartrate	0.22
Cellulose/Pectin	6.00
Lipid	
Corn oil diet	15
Fish oil diet	
Corn oil	3.5
Fish oil	11.5
Total	100

[1] given in Table A-1.
[1] given in Table A-2.

docosahexaenoic acid (DHA, 22:6n-3) (11.3%) in fish oil and higher concentration of linoleic acid (18:2n-6) in corn oil compared to the fish oil (55.4% versus 0.6%). The fish oil diets contained 3.5 g of corn oil per 100 g of diet to ensure the necessary level of linoleic acid. Additionally, 0.015 g of α-tocopherol (MT-70, Archer Daniels Midland, Decatur, IL) and 0.005 g of 20% tertiary butylhydroquinone (20% TBHQ, Gillco Ingredients, Vista, CA) were added per 100 g of fish oil diet as antioxidants. Corn oil diets were supplemented with TBHQ (0.019 g / 100 g of diet) to obtain antioxidant levels equivalent to that in the fish oil diets.

All liquid diet ingredients were mixed together, added to the relevant basal diets and intermixed until homogeneous. Fresh diets were prepared as necessary and placed in the dark at -20^0C for long-term storage (months) or at -4^0C for the short-term storage (weeks) to prevent the formation of oxidized lipids.

2.4. IRON IRRADIATION

When six weeks old, after the rats had been receiving the administered diet for three weeks, half of them (120 animals) were exposed to a single dose of approximately 1 Gy Fe-56 ions delivered with a dose rate from 0.49 ± 0.07 to 0.75 ± 0.22 Gy/min (mean$\pm$SD). The exposures were performed at the Alternating Gradient Synchrotron/Relativistic Heavy Ion Collider (AGS/RHIC) facility, Brookhaven National Laboratory (BNL, Upton, NY).

Accelerator operation was performed prior to the animal irradiation to ensure the necessary exposure level could be accurately reached and maintained.

Poly-methyl-methacrylate rat holders were used to immobilize animals during the irradiation procedure. They represent semicylinders with 3 mm thick walls having a set of ventilating holes in one of the semicylinder end and a slide damper plate at the other end. The rats were placed into the rat holders just prior to the radiation exposure and mounted on the supporting plexiglass block for stability. Animals were irradiated laterally, two at a time. They were returned to their individual cages immediately after the radiation exposure and given three days of recovery period before being shipped to LARR, TAMU.

2.5. CARCINOGEN TREATMENT

Azoxymethane (AOM, Sigma-Aldrich Corp., St. Louis, MO) was used as a targeted colon carcinogen and was administered by subcutaneous injection. The first dose of AOM was injected 32 days after the rats started receiving the experimental diets (ten days after the radiation exposure for the irradiated groups). The second dose of AOM was injected one week after the first one. In both cases the injection volumes were adjusted to deliver 15 mg/kg body weight.

2.6. HISTOLOGY SAMPLE PREPARATION

At the time of sacrifice, animals were visually examined and palpated from the outside and all subcutaneous mass lesions were resected and fixed in formalin.

The abdominal wall was then dissected; the entire colon and small intestine were removed. After removal of the rectum, the most distal 1 cm of colon was resected and designated “distal colon”. The first 1 cm of colon at the cecum/proximal colon junction was also resected and designated “proximal colon”. The colon was then flushed with phosphate-buffered saline and opened longitudinally. All macroscopic tumors were charted (location, size (length, versus width, versus depth) and the distinctive features of the tumors (color, firmness, homogeneity, if applicable) were noted.

The first 1 cm of small intestine at the ileocecal junction was resected and defined as the distal end of the ileum. Ten centimeters distally from this point, the ileum was resected. The first 1 cm from the pylorus of the stomach was resected and defined as the proximal end of the duodenum. Ten centimeters distally from this point the duodenum was resected. The section between the duodenum and ileum was defined as jejunum.

It should be noted that the sections of small intestine being resected during the experiment did not match in length with conventional small intestine segments: anatomically the ileum is shorter and the duodenum is longer than the sections taken. This was caused by the need for the defined quantities of tissues for parallel experiments.

All three sections of the small intestine were flushed with phosphate-buffered saline and examined from the outside. All macroscopic tumors found at ileum, jejunum and duodenum were charted as previously described except for the exact tumor location and the distinctive features of the tumors were noted.

All the suspected tumor tissue samples resected were then fixed depending on their size and location. Sessile colon and small intestine potential tumors of length/width less than 1 cm were fixed in 4% polyformaldehyde (PFA) for 4 hours. Sessile colon and small intestine potential tumors of length/width more than 1 cm and pedunculated colon and small intestine potential tumors were split and fixed in 4% PFA for 4 hours and 70% ethanol (EtOH). All subcutaneous tumors were fixed in 37% polyformaldehyde (formalin). They were then processed in VAPH Histology Laboratory, TAMU and embedded into paraffin blocks for long-term storage. For all lesions 4 µm sections were made perpendicular to their surface and stained with hematoxylin and eosin (H&E). Serial sections were cut whenever needed to expose the central part of the tumor, its stalk (if present) or microscopic tumor foci at the areas of focal atypia, as characterized by pseudostratification of epithelial cells and their orientation along sinus basement membranes. Tumors were classified as adenomas or adenocarcinomas as previously described (99).

CHAPTER III

RESULTS

3.1. GENERAL OBSERVATIONS

3.1.1. Histological observations

Single or, rarely, multiple cell aggregations located within lymphoid nodules below the muscularis mucosae were found in colon tissue samples of all diet and irradiation groups. These aggregations were composed of epithelial cells expressing slight dysplasia and mainly resembling glandular structures. In these cases an increased mucin production with mucous pools formation within dilated crypts was common. No mitotic activity was noted in the lining epithelium. Cellular or nuclear pleomorphism, nuclear hyperchromatism, loss of nuclear polarity were not present. No prominent stromal desmoplastic reaction was noted in most of the cases.

To determine if the aggregate formation was related to the AOM injection, a total of 108 reference H&E stained normal colon tissue samples were microscopically examined. These samples were resected from rats of the same strain, which were consuming the same experimental diets, but injected with saline instead of the AOM, and euthanized when 39 weeks of age. Of these samples, 12 (11%) showed presence of similar epithelial structures within the lymphoid nodules. As long as no histological changes that would point to the neoplastic nature of these aggregates were present, they were defined as “possible preneoplastic lesions” and were not considered further.

3.1.2. Food intake

Two food intake measurements were performed during the study: before the initial AOM injection (38 days after the rats started receiving the experimental diets and ten days after the radiation exposure for the irradiated groups), and 20 weeks after the second AOM injection (Fig. 1).

Fiber was found to be a significant factor affecting food consumption at the first time point (Fig. 2A). However, when the two radiation treatment groups were considered separately, the effect of fiber on the food consumption was significant for non-irradiated animals only (P=0.0014) with the cellulose-based diet intake dominating over the pectin-based one (Fig. 2B). When a diet was the factor of interest, it was found to be a significant factor affecting food intake (P=0.0381), where fish oil/pectin diet consumption was found to be markedly lower than that of corn oil/cellulose and fish oil/cellulose diets (P=0.0117 and P=0.0398, respectively). Again, when both radiation treatment groups were considered separately, the effect of diet on food consumption was significant for non-irradiated animals only (Fig. 3B).

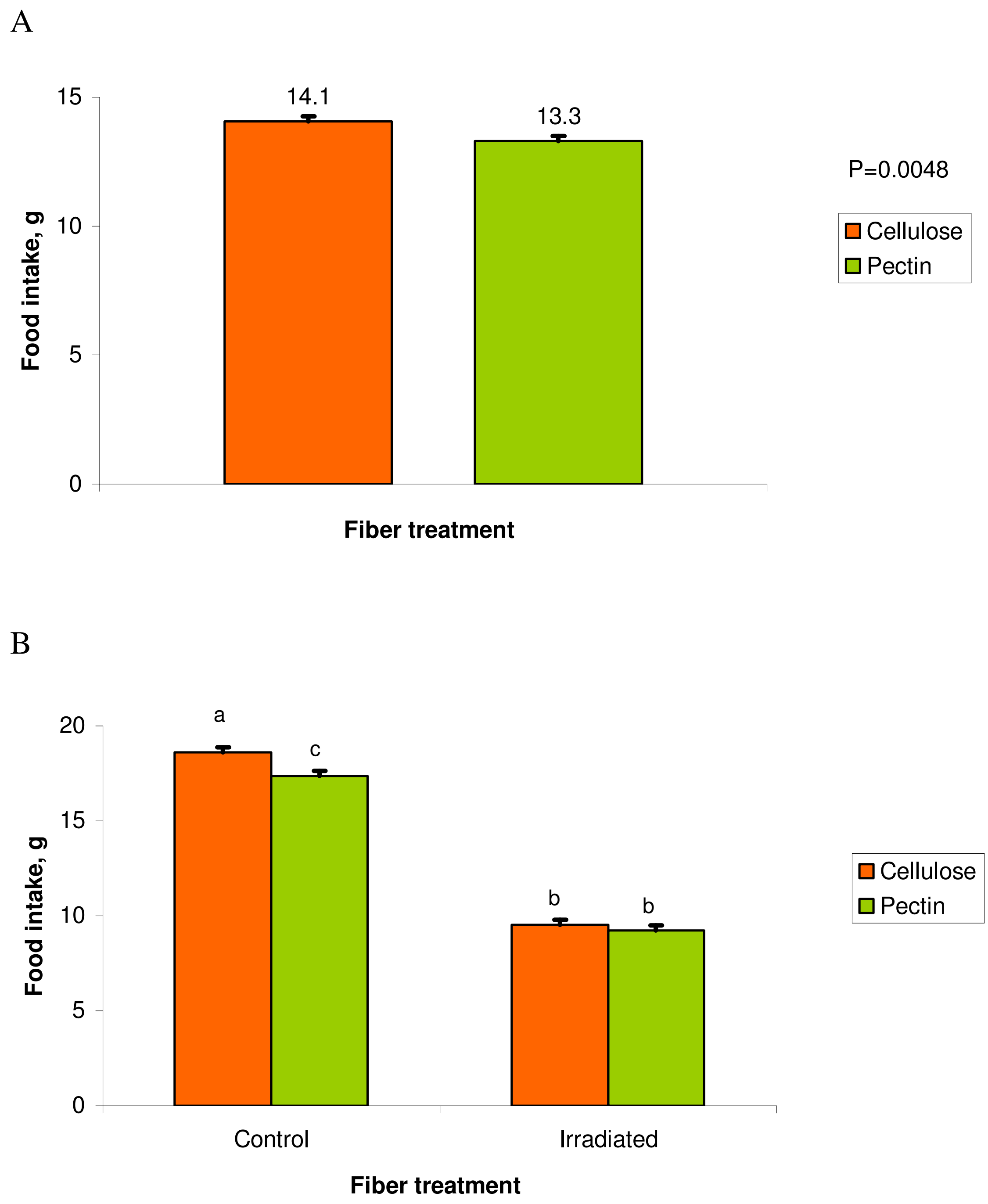


Fig. 2. Food intake measured before the 1st AOM injection, fiber treatment group comparisons. General *(A)* and with irradiation considered *(B).* Data presented as LS means $\pm$ SE.

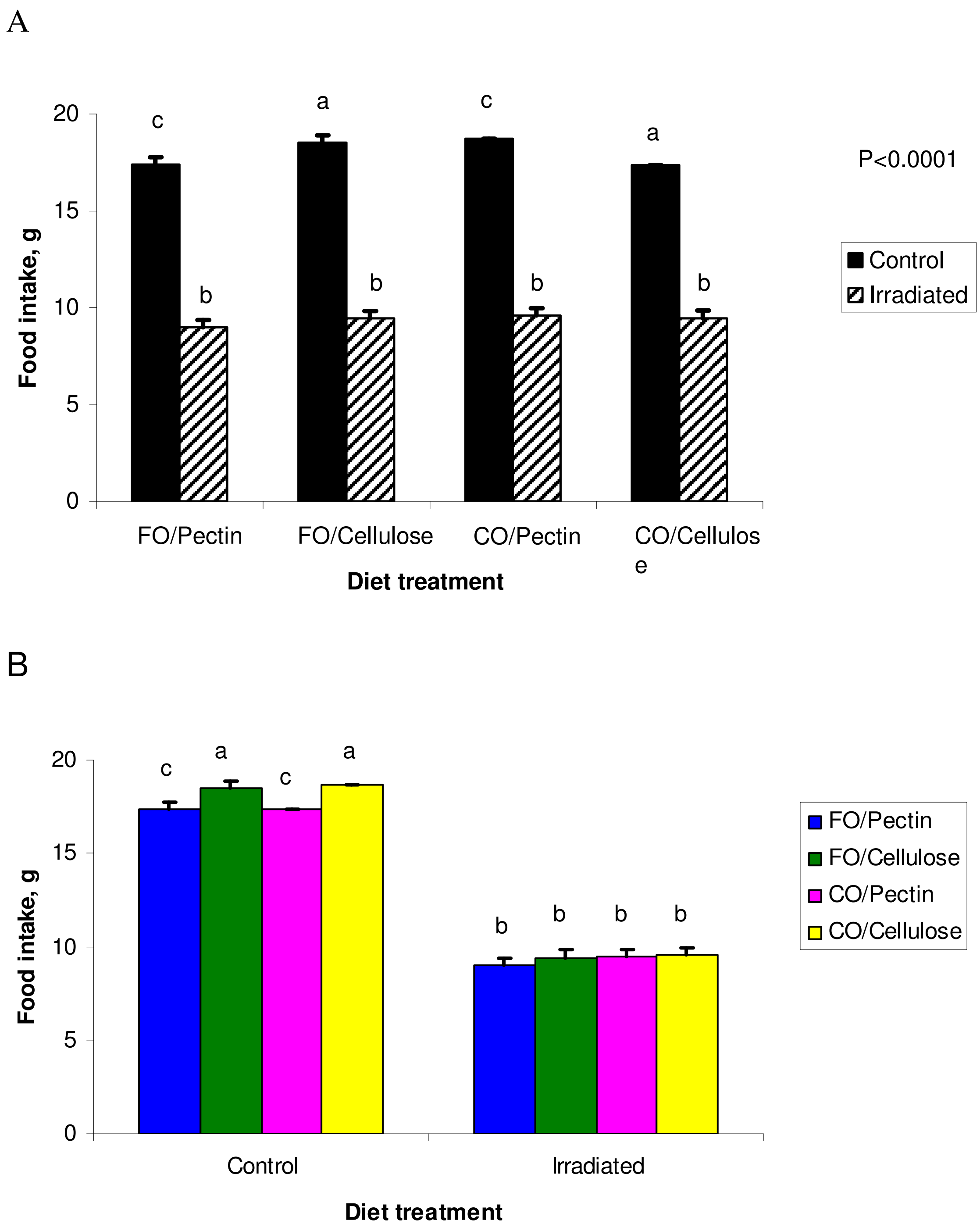


Fig. 3. Food intake measured before the 1st AOM injection. Irradiation *(A)* and diet treatment *(B)* group comparisons. Data presented as LS means $\pm$ SE.

Similarly, fiber was found to be a significant factor affecting food consumption at the second food intake measurement (Fig. 4A). Separate analyses of the second food intake data showed no significant effect of fiber on the food consumption, though for the non-irradiated group of rats the consumption of the cellulose-based diet was still higher than that of the pectin-based one (P=0.0550) (Fig. 4B). When the diet was a factor of interest, it was found to be a significant factor affecting food intake (P=0.0130), where fish oil/cellulose diet consumption was found to be markedly higher than that of corn oil/pectin and fish oil/pectin diets (P=0.0018 and P=0.0173, respectively). Again, when two radiation treatment groups were considered separately, the effect of diet on the food consumption was different for non-irradiated vs. irradiated animals (Fig. 5B) with a prevalence of corn oil cellulose over corn oil/pectin diet consumption within the non-irradiated group and of fish oil/cellulose over other diets consumption within the irradiated group of animals.

Marked decrease of food intake was found in the irradiated groups when compared to the non-irradiated groups at both time points (Figs. 3A and 5A). At the first food intake, irradiated rats were shown to consume 1.9 times less experimental diet than non-irradiated rats (P<0.0001). The second food intake was found to be on average 1.2 times higher for the non-irradiated group vs. the irradiated one (P<0.0001).

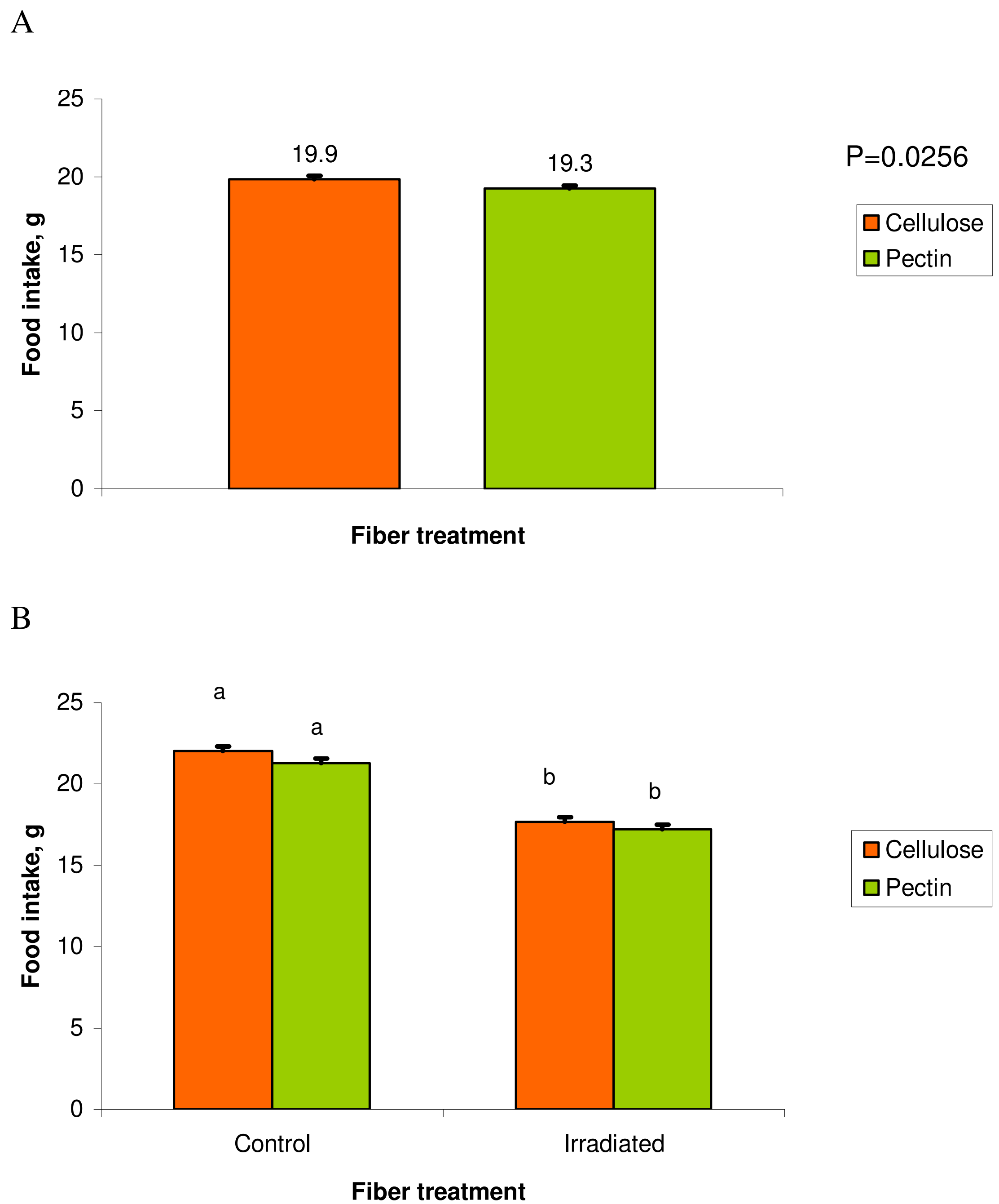
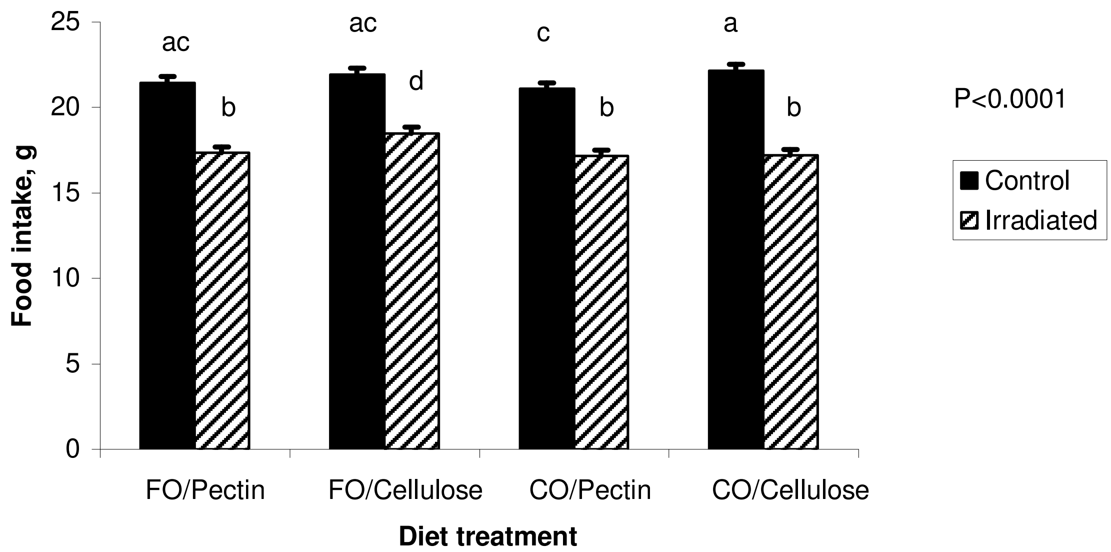


Fig. 4. Food intake measured 20 weeks after the 2nd AOM injection, fiber treatment group comparisons. General *(A)* and with irradiation considered *(B)*. Data presented as LS means + SE.

A



B

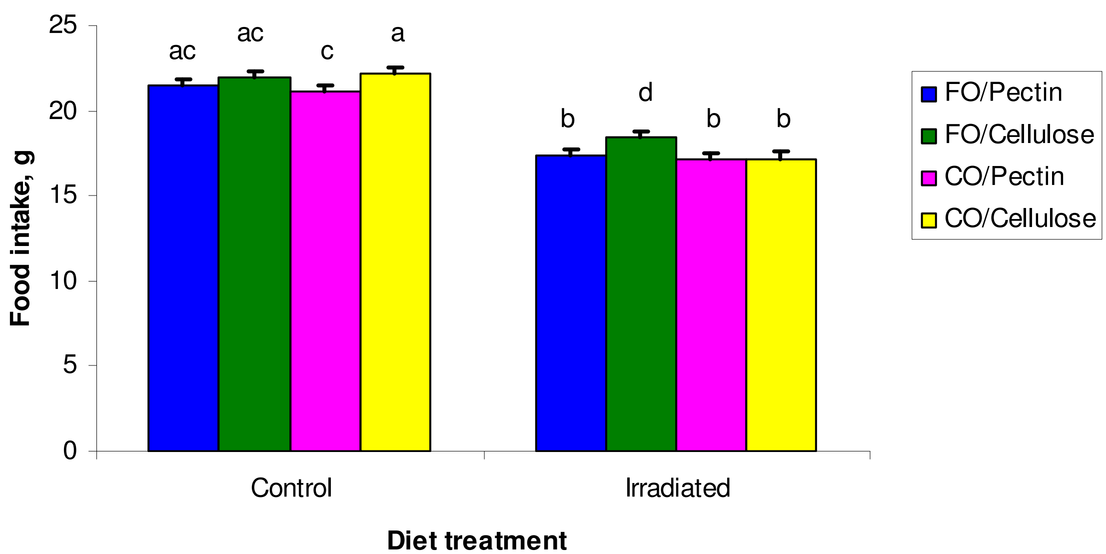


Fig. 5. Food intake measured 20 weeks after the 2nd AOM injection. Irradiation *(A)* and diet treatment *(B)* group comparisons. Data presented as LS means $\pm$ SE.

3.1.3. Body weight and weight gain

Body weights and weight gain of all diet and irradiation group representatives were compared (see Tables B-1 and B-2 of Appendix B for details). Weight gain data for two irradiated and two non-irradiated groups of rats were combined together and the difference in kill times was eliminated using the kill time as a covariate. Body weight data could not be combined because of significant interaction of the covariate and the main effect.

It was found that animals exposed to Fe-ion radiation were gaining less weight ($P<0.0001$) (Figs. 6A and 7A) to the both intermediate and final weight gain checkpoints. Consequently, they had lower body weights to all three time points after the diet assignment, though at the beginning of the experiment their body weights were slightly higher in comparison with those of non-irradiated rats (Fig. 8). However, when two irradiated and two non-irradiated groups were considered separately, the difference in body weights was significant as between non-irradiated groups as between irradiated ones.

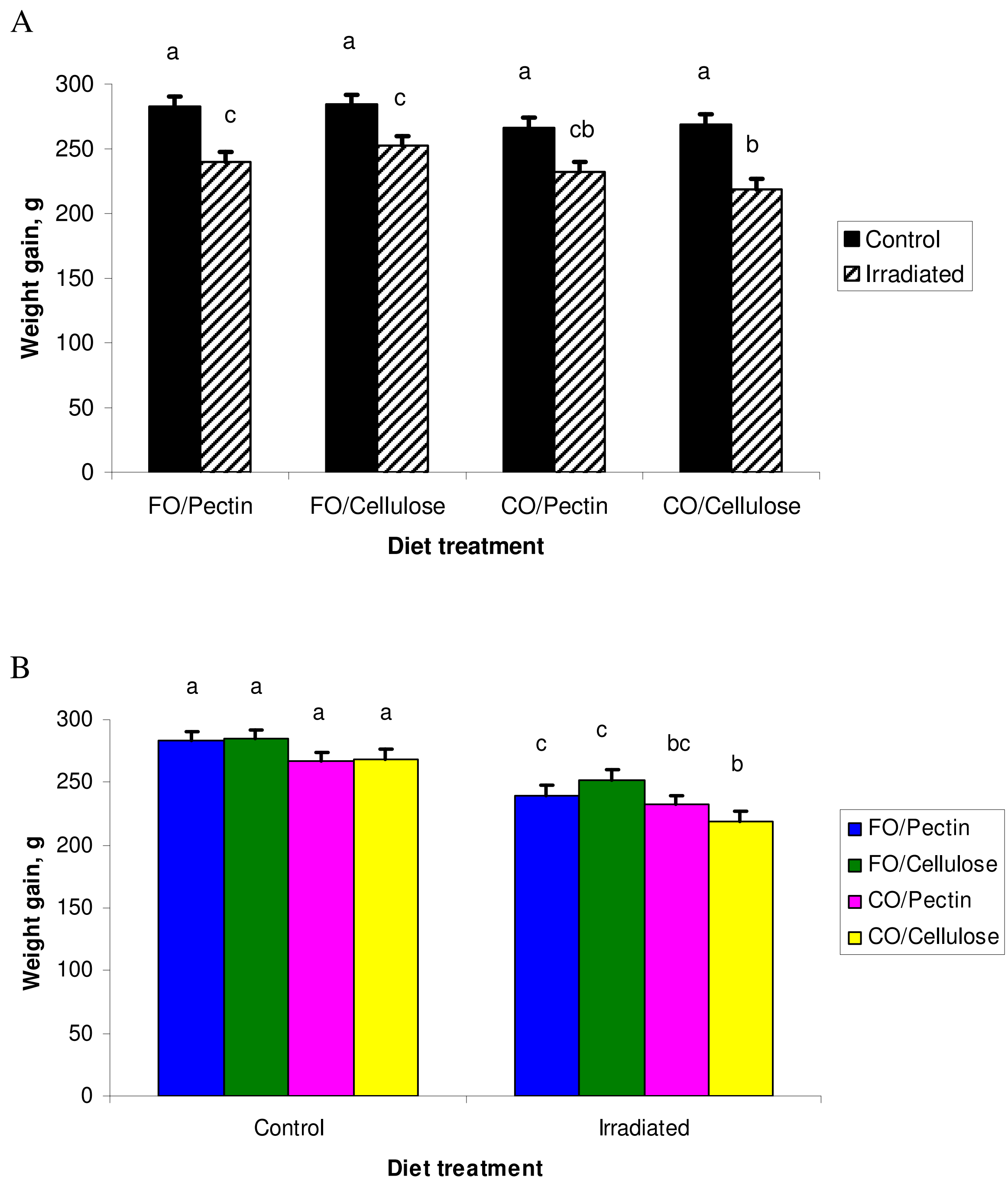
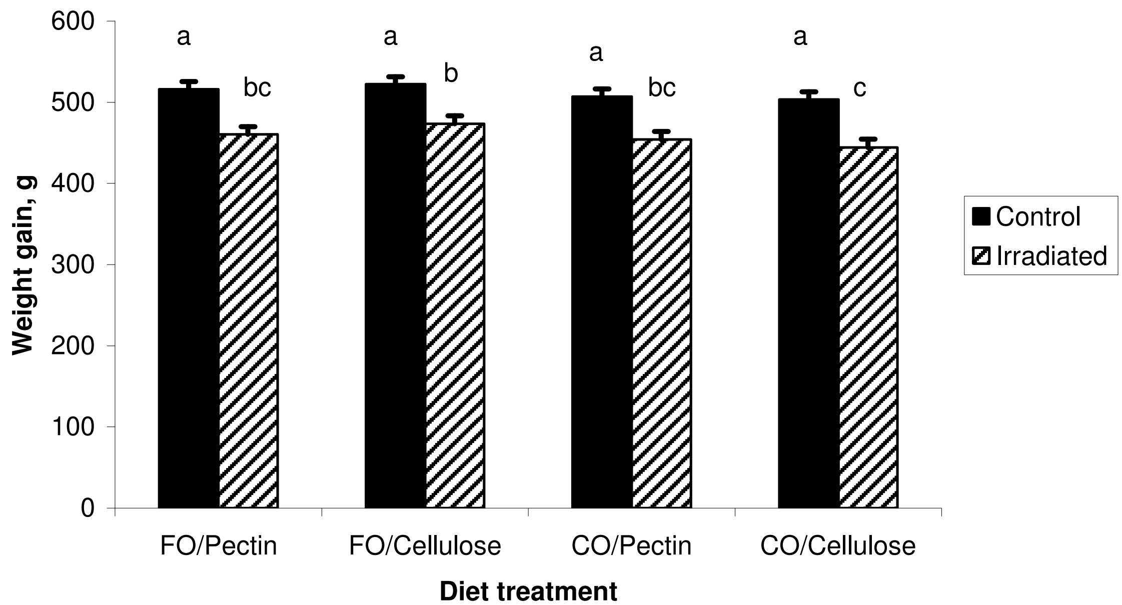


Fig. 6. Intermediate weight gain. Irradiation *(A)* and diet treatment *(B)* group comparisons. Data presented as LS means $\pm$ SE.

A



B

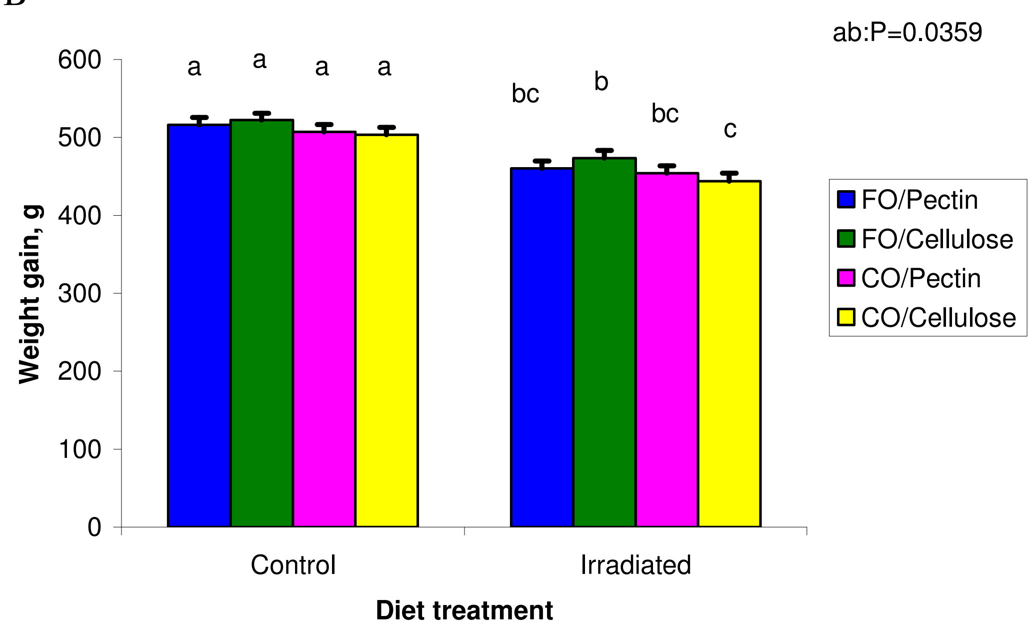


Fig. 7. Final weight gain. Irradiation *(A)* and diet treatment *(B)* group comparisons. Data presented as LS means ± SE.

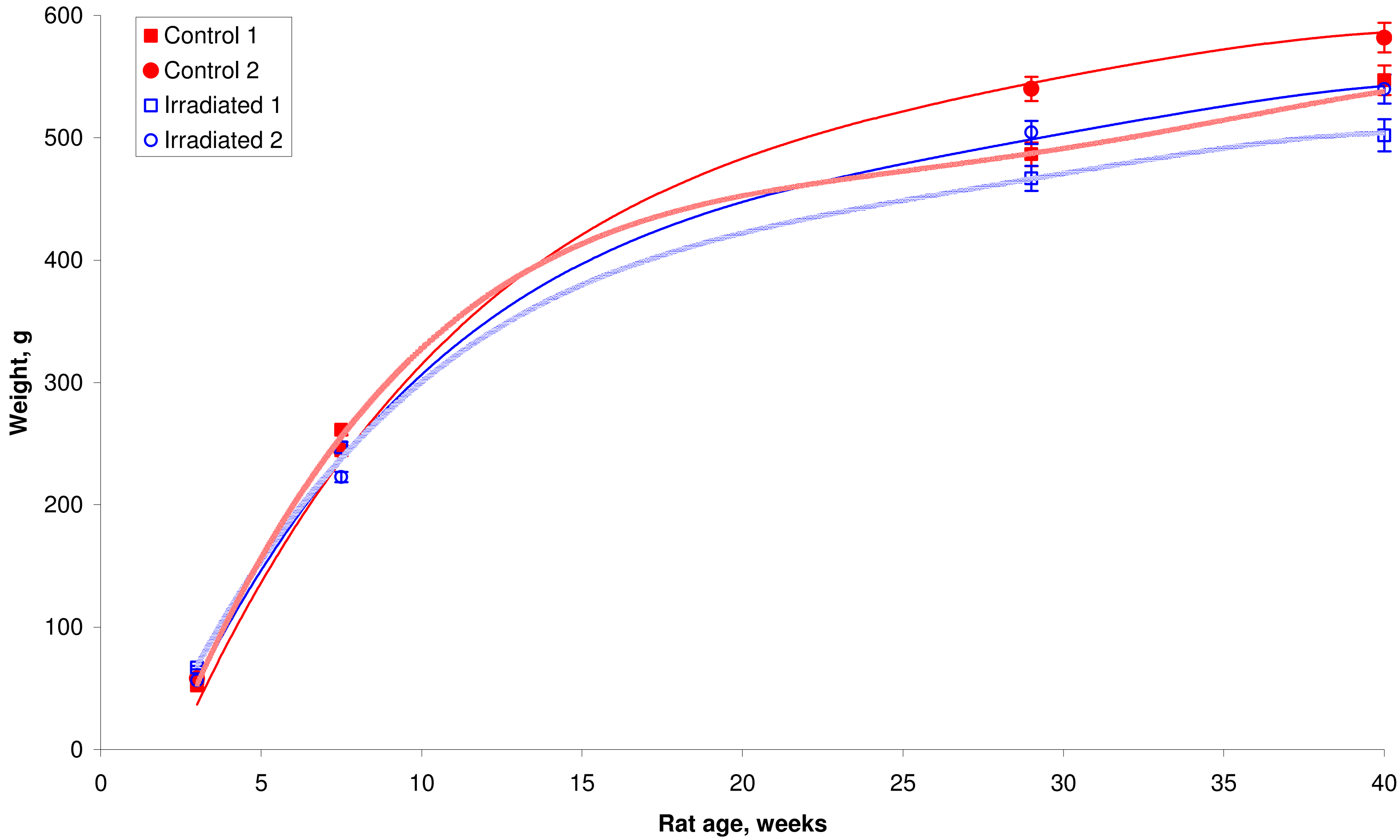


Fig. 8. Rat body weight, four irradiation group comparisons. Data presented as LS means ± SE.

Between groups received different radiation treatment the difference was significant for non-irradiated group 1 and irradiated group 2 when compared (Fig. 9). No significant weight differences between diet groups within four and, consequently, when combined, two radiation treatment groups were noted at the first two weight checkpoints (Figs. 10, 11, 12 and 13).

There were differences in body mass gained to the third and to the final, the fourth, time points. They reflected on both intermediate and final weight gain data (Figs. 6 and 7) and, consequently, on body weights. Body weight differences were found as between groups received different radiation treatment (Fig. 9), as within groups received the same radiation treatment (Fig. 8), as between different intervention diet groups (Figs. 10, 11, 12 and 13). It should be noted, however, that, when two irradiated and two non-irradiated groups data were combined, no significant difference between diet groups within groups received the same radiation treatment was found.

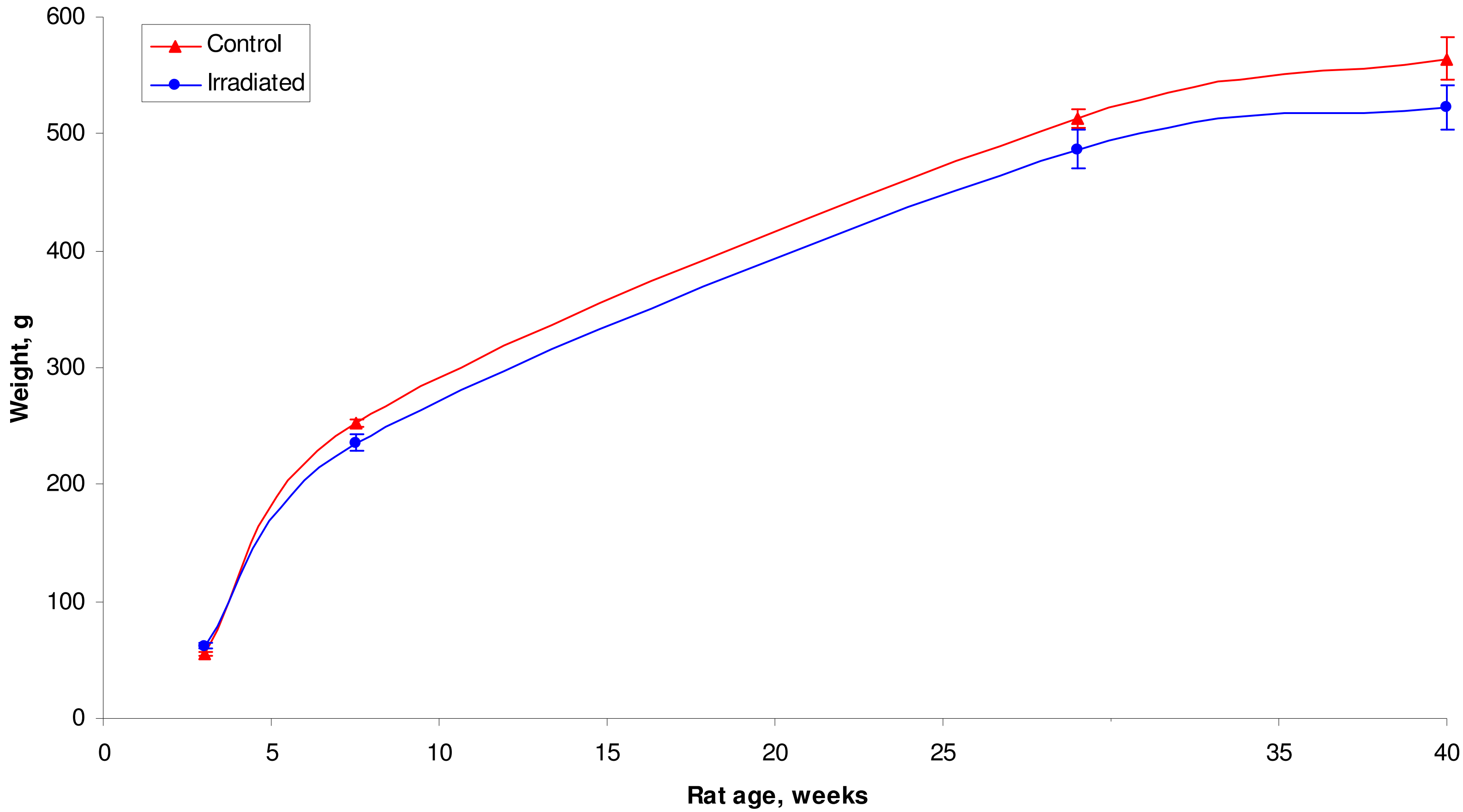
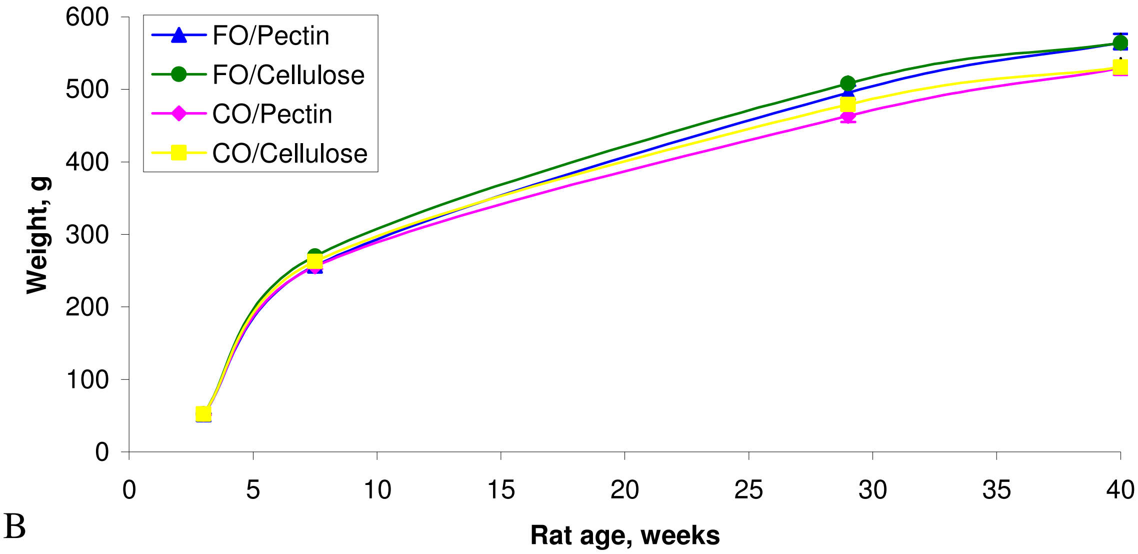


Fig. 9. Rat body weight, two irradiation group comparisons. Data presented as LS means ± SE.

A



B

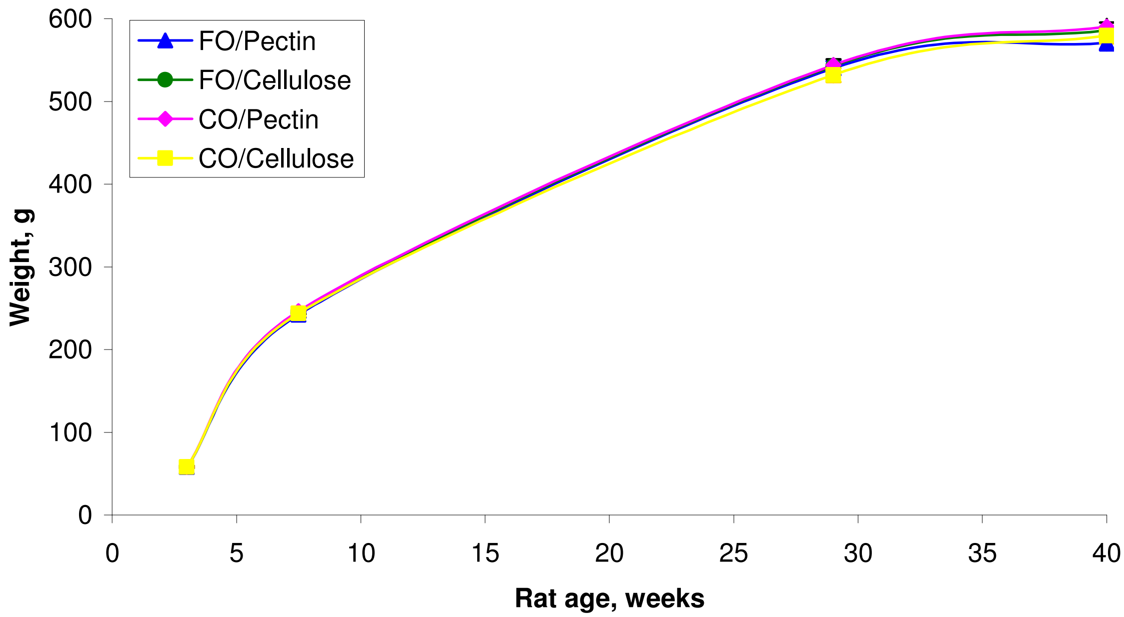
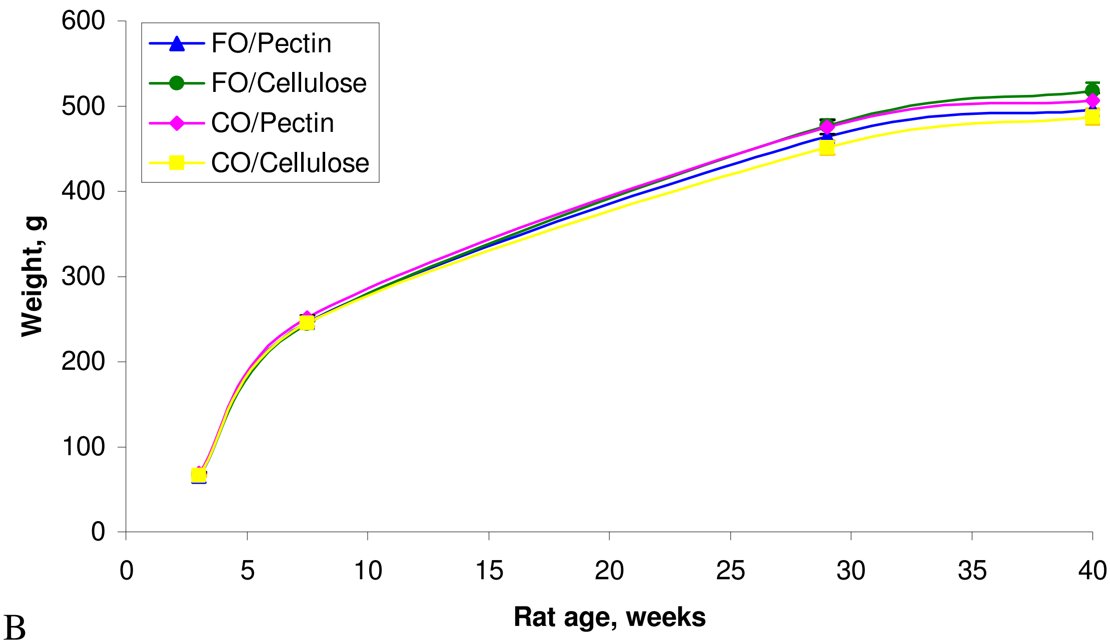


Fig. 10. Rat body weight, non-irradiated groups 1 *(A)* and 2 *(B)*. Diet treatment group comparisons. Data presented as LS means ± SE.

A



B

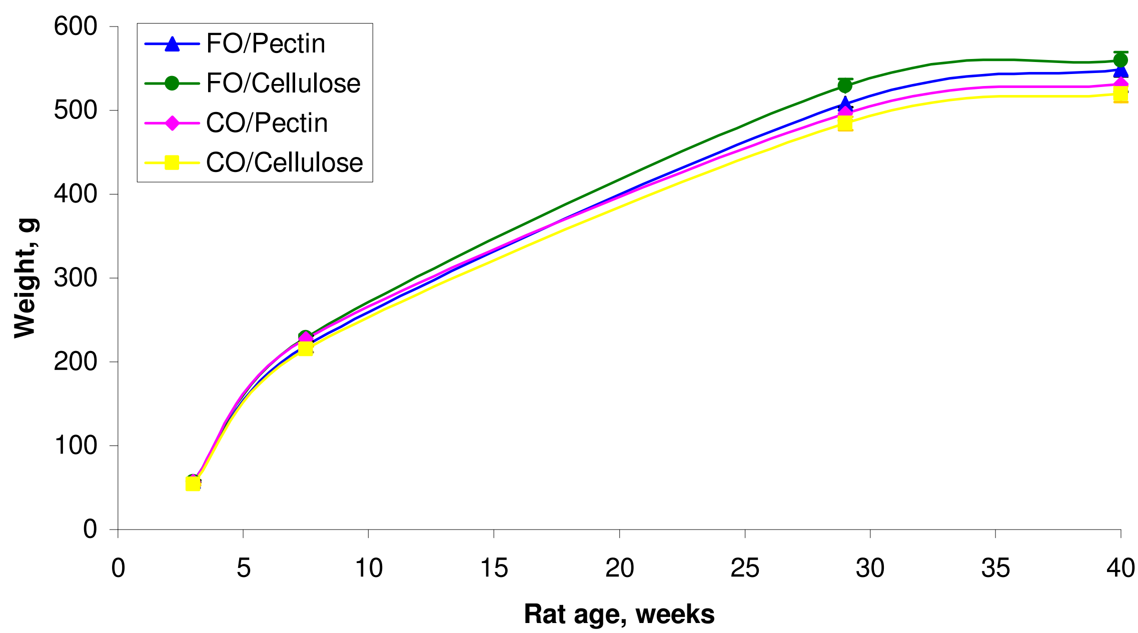


Fig. 11. Rat body weight, irradiated groups 1 *(A)* and 2 *(B)*. Diet treatment group comparisons. Data presented as LS means $\pm$ SE.

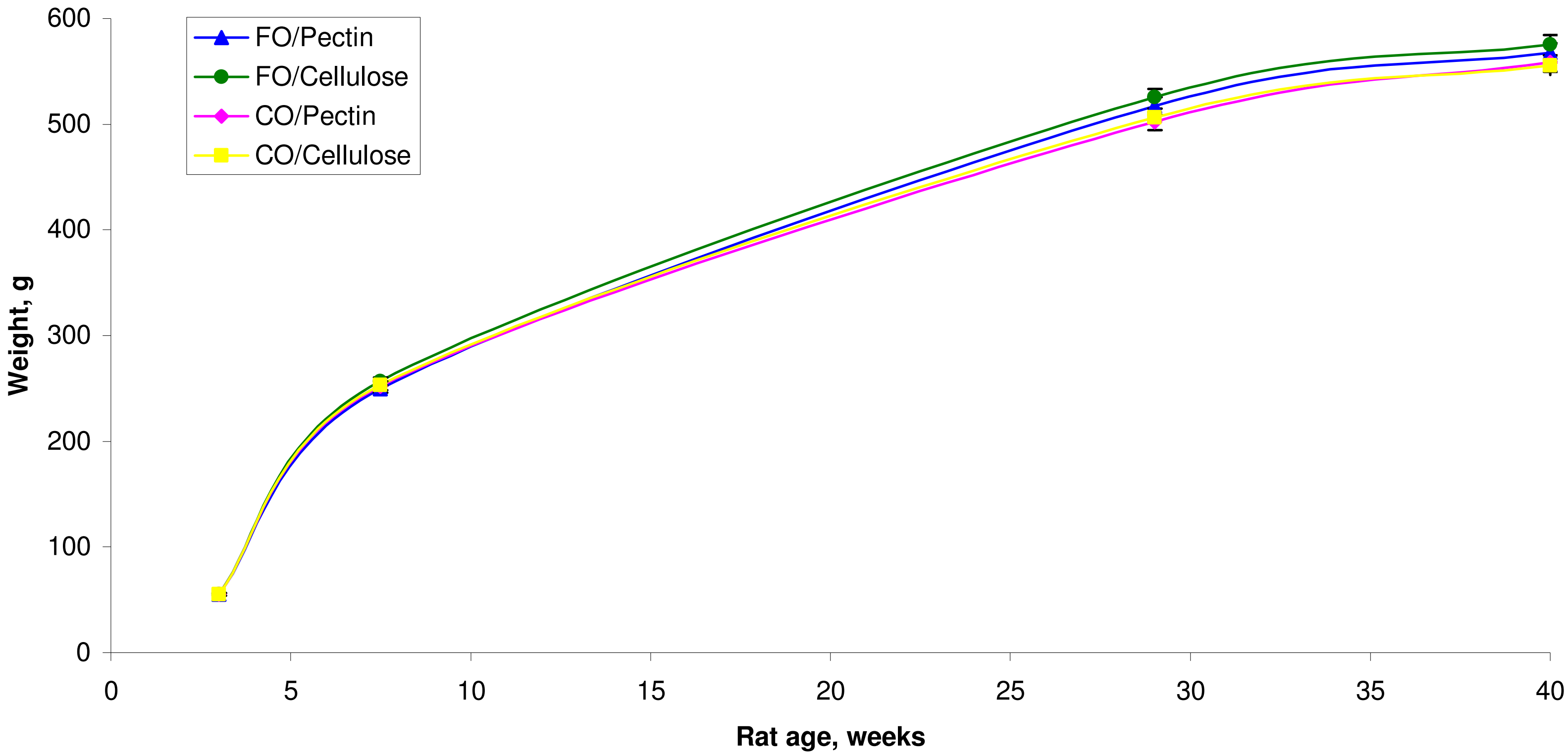


Fig. 12. Rat body weight, non-irradiated group. Diet treatment group comparisons. Data presented as LS means ± SE.

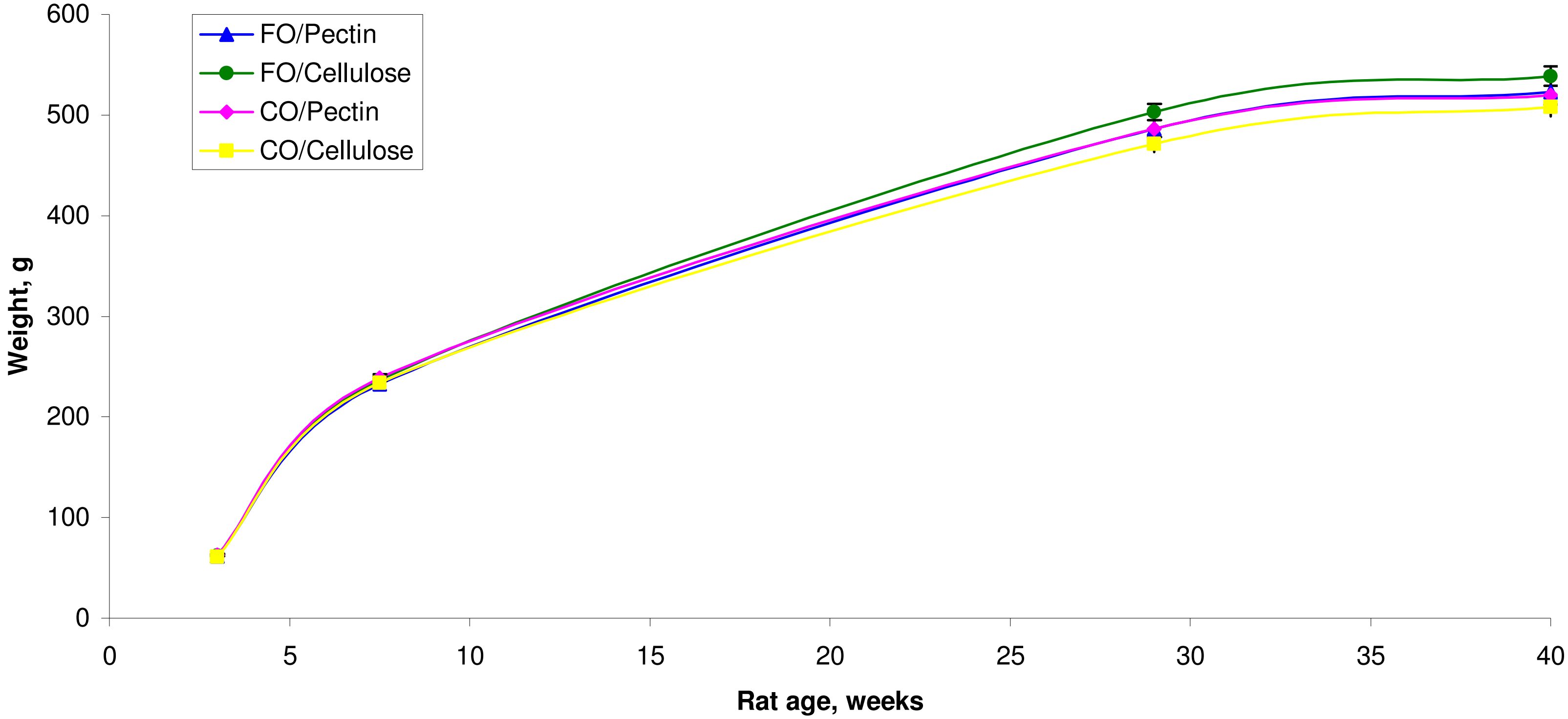


Fig. 13. Rat body weight, irradiated group. Diet treatment group comparisons. Data presented as LS means ± SE.

3.2. MORBIDITY AND MORTALITY

There were unexpected losses of animals in both control and irradiated groups, 5 and 21, respectively (4% and 17%). Several rats were found dead in their cages at different time points. As it has already been said, any animal expressing signs of sickness such as lethargy, rectal or outer tumor bleeding, continuous weight loss, continuous diarrhea, not eating, seizures or problems with locomotion was euthanized at that time. The animals lost from the experiment this way were included in the mortality study. All the rats lost before the end of the experiment were divided into groups according to the fiber, oil and diet type and also radiation exposure. Survival functions were obtained for these groups according to Kaplan-Meyer estimation (100).

There was a marked decrease in survival rate in the irradiated group when compared to the non-irradiated group of rats at the main kill time point, 31 weeks after the 2nd AOM injection (81.6% vs. 95.8%, P=0.0005, see Fig. 14). Irradiated animal losses (including necessary euthanasia) began 11 weeks earlier than those of non-irradiated animals.

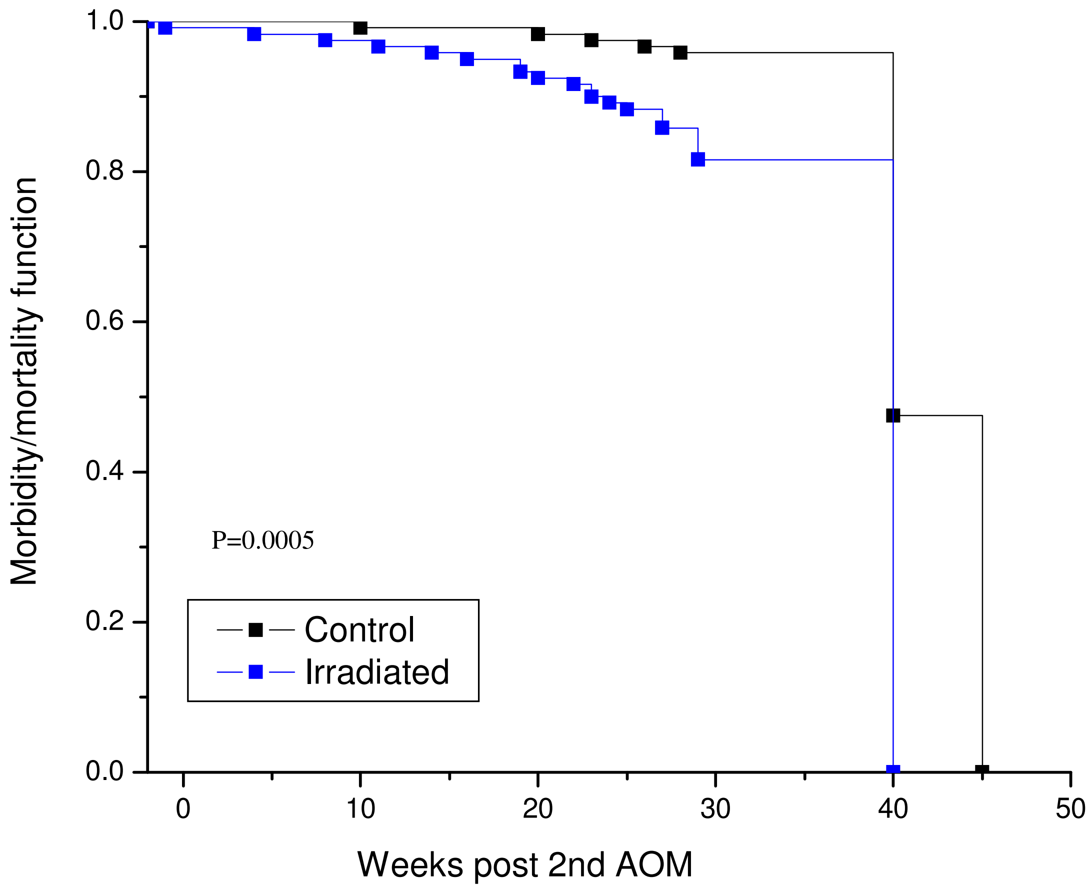


Fig. 14 Morbidity/mortality curves for different radiation treatment groups of rats.

It was also found that survival rate was dependent on diet components. Thus, animals consuming fish oil-based diets seemed to have a longer lifespan when compared to those consuming corn oil-based diets for both irradiated and non-irradiated groups (10- and 20-week difference for non-irradiated and irradiated animals, respectively, see Fig. 15). Similarly, pectin was found to be a more beneficial fiber type in comparison with cellulose with respect to the lifetime for both radiation treatment groups (10- and 12- week difference for non-irradiated and irradiated animals, respectively, see Fig. 16). The percent of animals surviving to the planned kill-time point was not found to be statistically different between diet and oil groups, though there was a higher survival tendency found for animals from pectin-based diet groups when compared to the cellulose-based diet groups (96.7% vs. 95.0% for non-irradiated and 85.0% vs. 81.0% for irradiated groups).

The combination of dietary oils and fibers also appeared to affect the morbidity/mortality (see Fig. 17). However, these data could not be analyzed statistically. Rats belonging to the corn oil/cellulose diet group had a tendency to exhibit the shortest lifespan when all the diet and radiation treatment groups were considered. The survival rate of this diet group was found to be the lowest among all the diet groups within the irradiated group (88.9%). For non-irradiated group animals, the survival rate of all diet groups followed the same pattern as for the fiber groups with survival of fish oil/cellulose being the lowest (96.7% at the main kill-time point). For irradiated animals, corn oil/pectin diet group had the highest survival rate among all diet groups (93.4%).

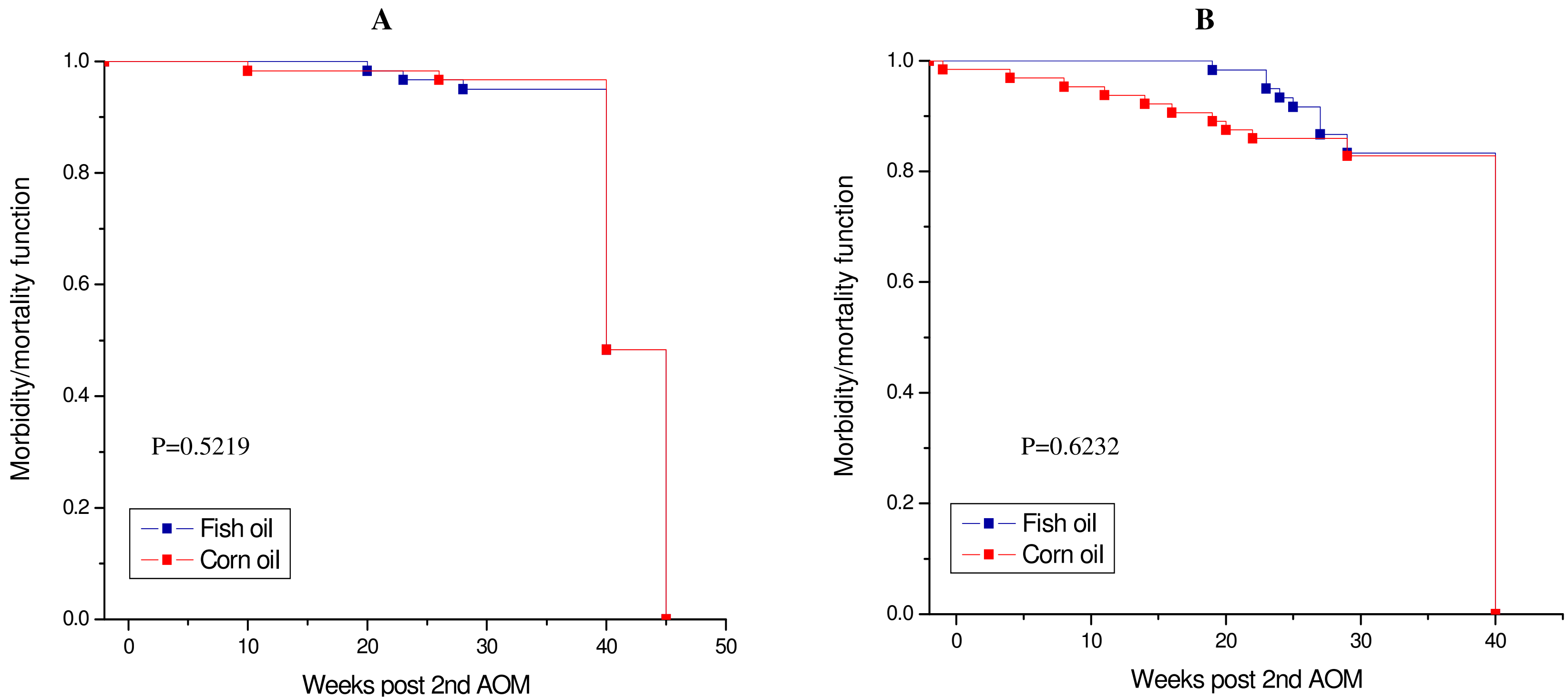


Fig. 15. Morbidity/mortality curves for different oil treatment groups of rats. *(A)* – non-irradiated rats and *(B)* – Fe-ion irradiated rats.

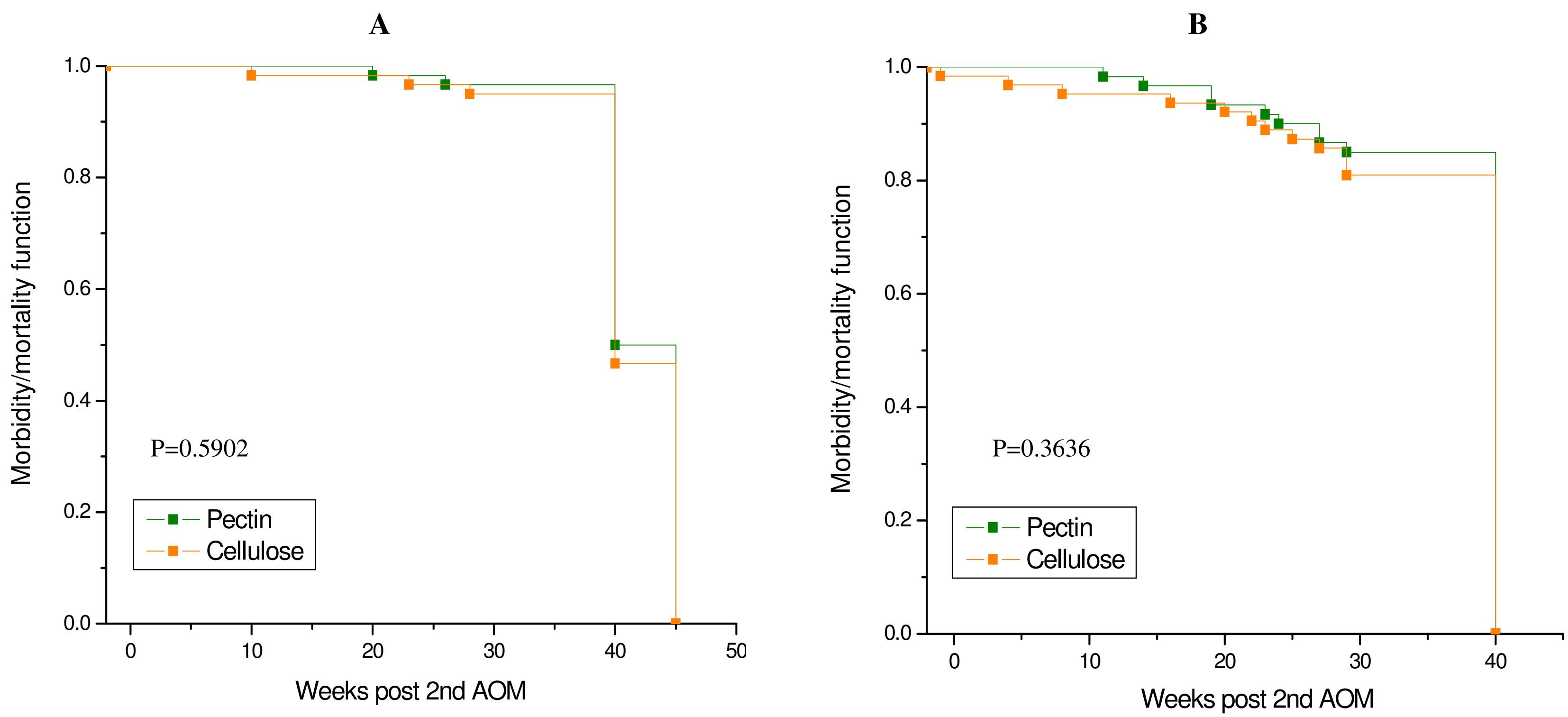


Fig. 16. Morbidity/mortality curves for different fiber treatment groups of rats. *(A)* – non-irradiated rats and *(B)* – Fe-ion irradiated rats.

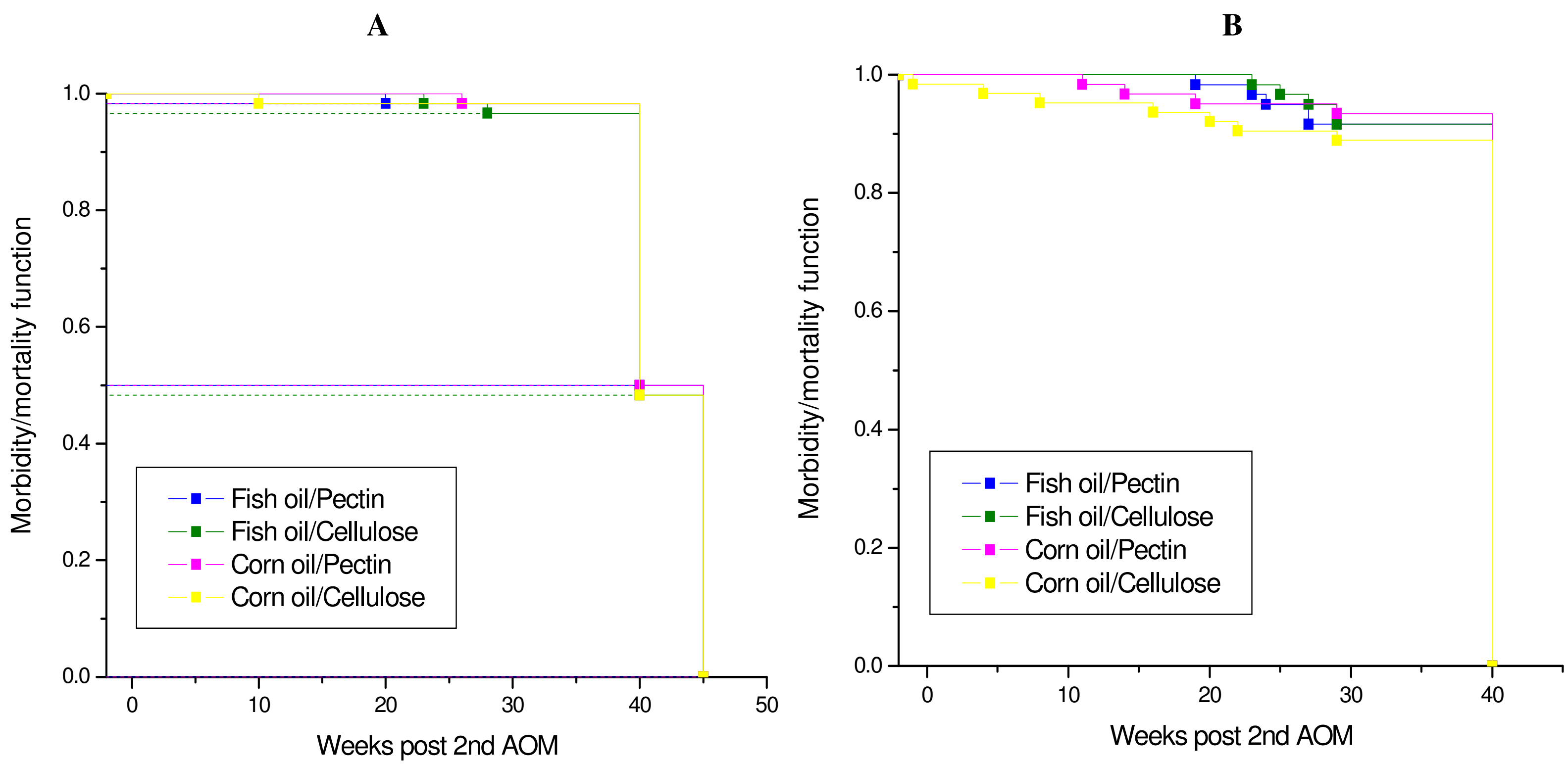


Fig. 17. Morbidity/mortality curves for different diet treatment groups of rats. *(A)* – non-irradiated rats and *(B)* – Fe-ion irradiated rats.

3.3. TUMOR INCIDENCE

Two separate analyses were made: using data from all animals in the study and then from the animals that survived to the end of study. Tabulated tumor incidence data are presented in Tables B-3 and B-4 of Appendix B.

Using the data from all animals used in the study, a cumulative tumor incidence graph was plotted as a function of time (any types of tumors at any location were considered) (Fig. 18). It was found that irradiated rats started developing tumors earlier than non-irradiated ones.

The effects of oil, fiber, radiation treatment and their possible combinations were then analyzed.

Oil was found to be a significant factor predicting the probability of having a colon tumor (P=0.0345 and P=0.0202 when all animals and only animals that survived to the end of the study were considered, respectively) (see Figs. 19B and 20B). All rats fed with corn oil-based diets were found to have 1.98 times higher colon tumor incidence than rats fed with fish oil-based diets, and the chances of having tumor for the corn oil-based diet group representatives increased to 2.2 times for the rats killed at the end of study (P=0.0345 and P=0.0202, respectively). However, when the two radiation treatment groups were considered separately, the effect of oil on colon tumor incidence was found to be significant for non-irradiated animals only (P=0.0244). No significant effect of fiber on colon tumor incidence was found. However, when combinations of oils and fibers were considered, all the rats consuming the corn oil/pectin diet had 2.12 times higher tumor incidence than rats consuming the fish oil/pectin diet, and, for the rats survived to the end of study, this ratio increased to 2.8 (P=0.0918 and P=0.0304, respectively).

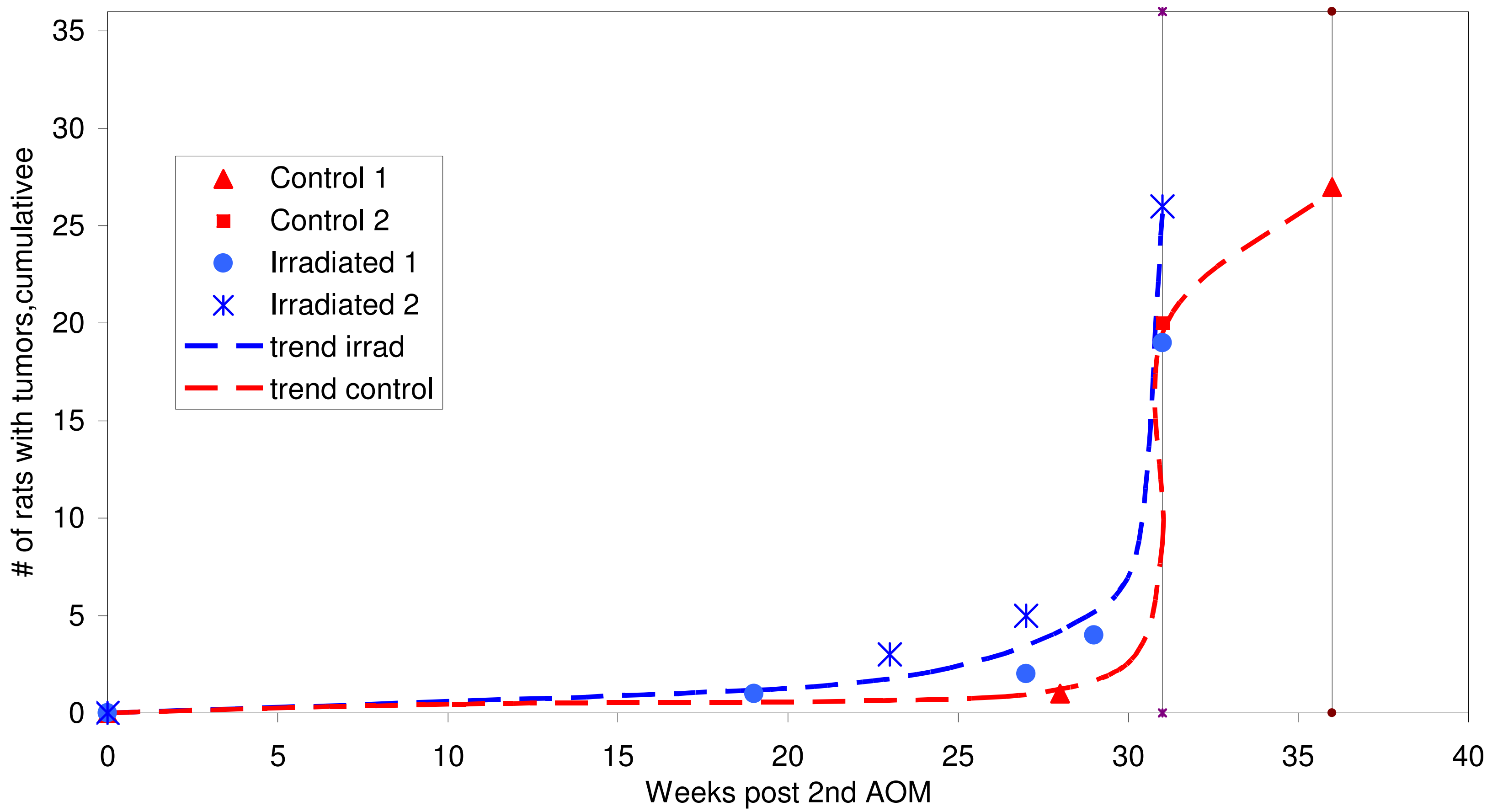
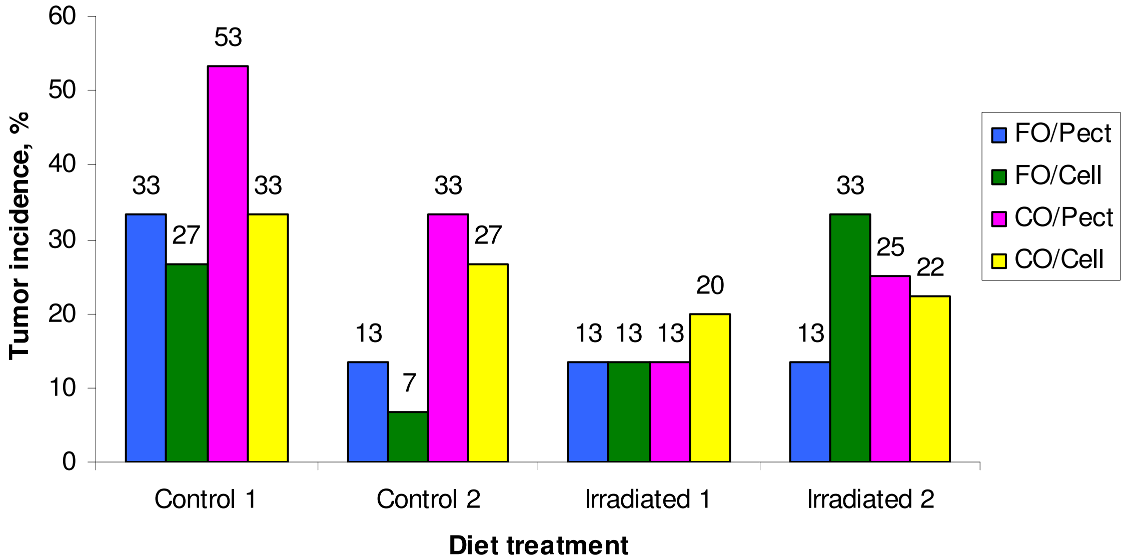


Fig. 18. Number of rats with tumors of any type and any location found, cumulative.

A



B

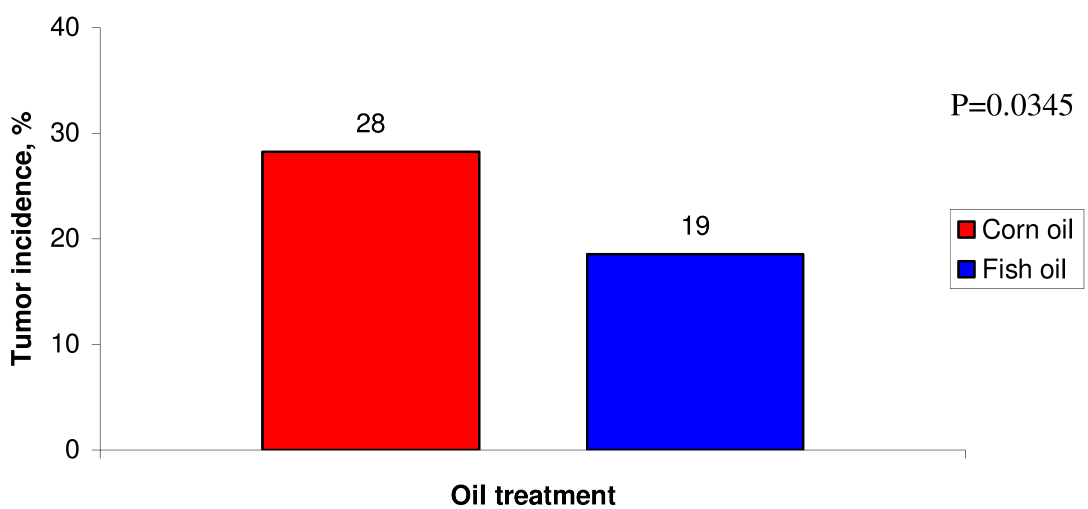
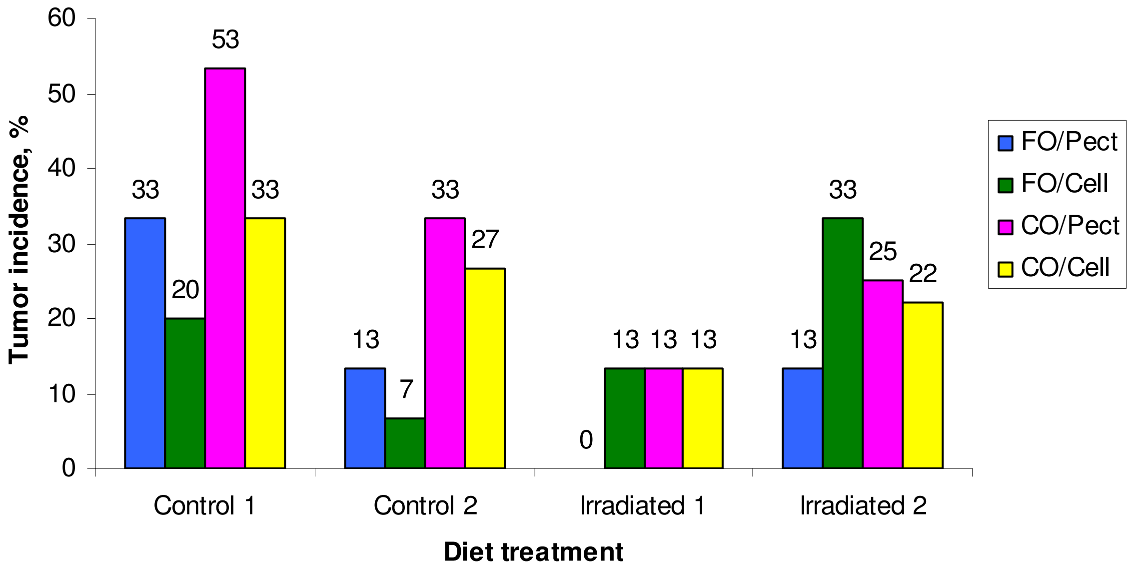


Fig. 19. Colon tumor incidence (all types of tumors), all animals included. Diet *(A)* and oil *(B)* treatment group comparisons.

Note: Control 1 group was euthanized 5 weeks later than other three groups of rats.

A



B

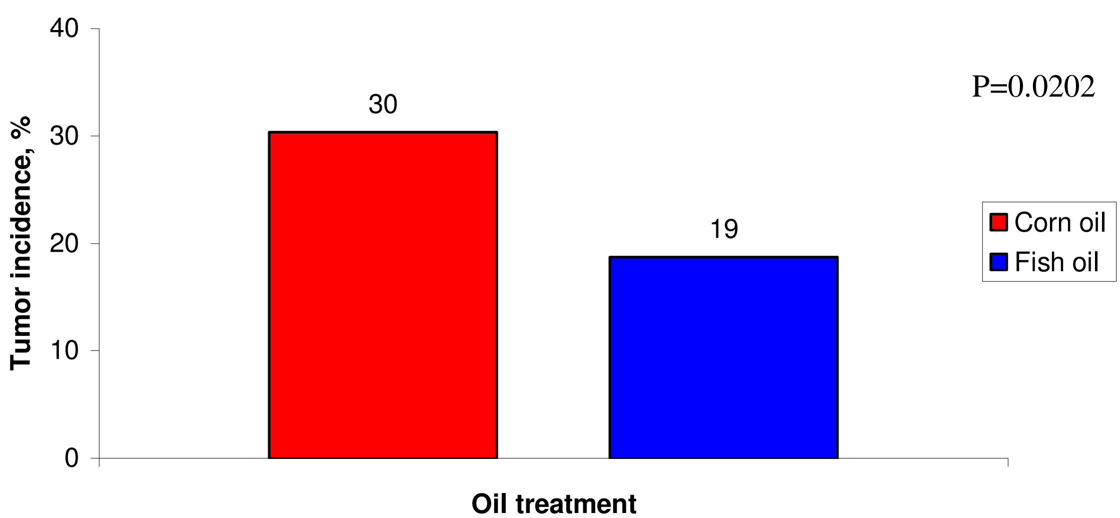
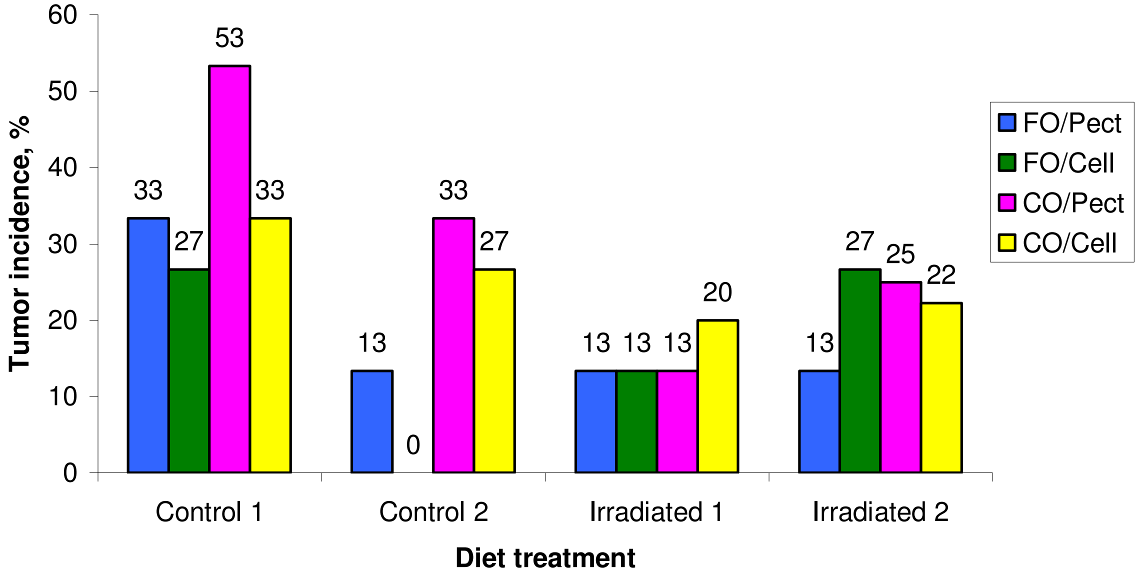


Fig. 20. Colon tumor incidence (all types of tumors), only animals survived to the end of study included. Diet *(A)* and oil *(B)* treatment group comparisons.
Note: Control 1 group was euthanized 5 weeks later than other three groups of rats.

No significant effect of radiation on colon tumor incidence was found. However, there was a clear difference found between irradiated vs. non-irradiated diet groups. Thus, when radiation treatment was not present, tumor incidence for corn oil/pectin fed rats appeared to be much higher than for fish oil/cellulose fed rats (P=0.0202) while, in the presence of radiation treatment, the lowest colon tumor incidence was found for fish oil/pectin fed rats (not statistically significant) (Figs. 19A and 20A).

When the malignant colon tumor incidence was examined, again, oil was found to be a significant factor. (P=0.0194 and P=0.0104 when all animals and only animals that survived to the end of the study were considered, respectively, see Figs. 21B and 22B). All the rats fed with corn oil-based diets were found to have 2.17 times higher colon tumor incidence than rats fed with fish oil-based diets, and the chances of having a tumor for the corn oil-based diet group increased to 2.46 times for the rats killed at the end of study (P=0.0194 and P=0.0104, respectively). However, when the two radiation treatment groups were considered separately, the effect of oil on malignant colon tumor incidence was found to be significant for non-irradiated animals only (P=0.0064). No significant effect of fiber on malignant colon tumor incidence was found. However, when combinations of oils and fibers were considered, all the rats consuming corn oil/pectin diet had a 2.13 times higher tumor incidence than rats consuming fish oil/pectin diet, and for the rats that survived to the end of study this ratio increased to 2.84 (P=0.0899 and P=0.0288, respectively). Similarly, the rats consuming corn oil/pectin diet had a 2.72 times higher tumor incidence than rats consuming fish oil/cellulose diet, and for the rats that survived to the end of study this ratio increased to 2.81 (P=0.0335 and P=0.0320, respectively).

A



B

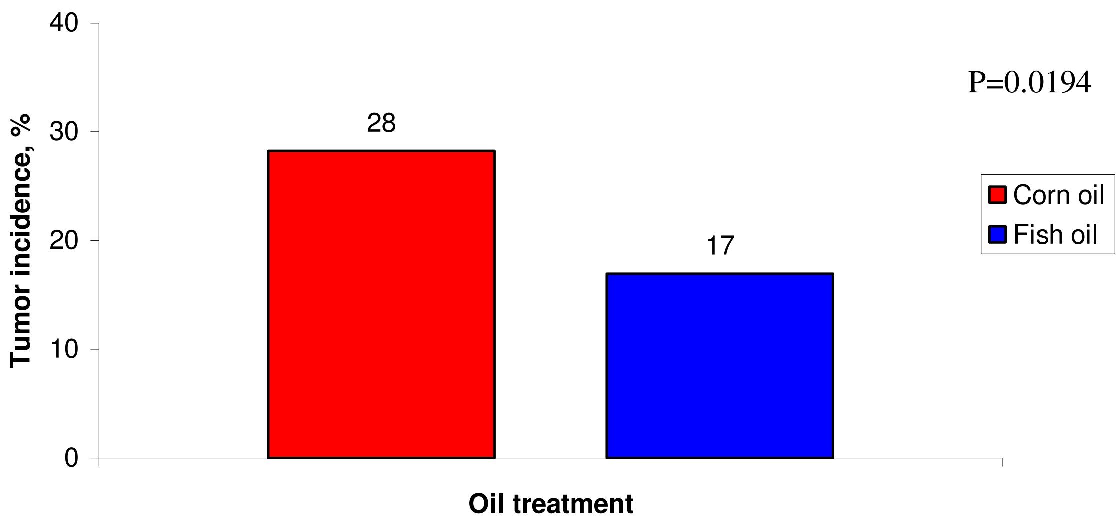
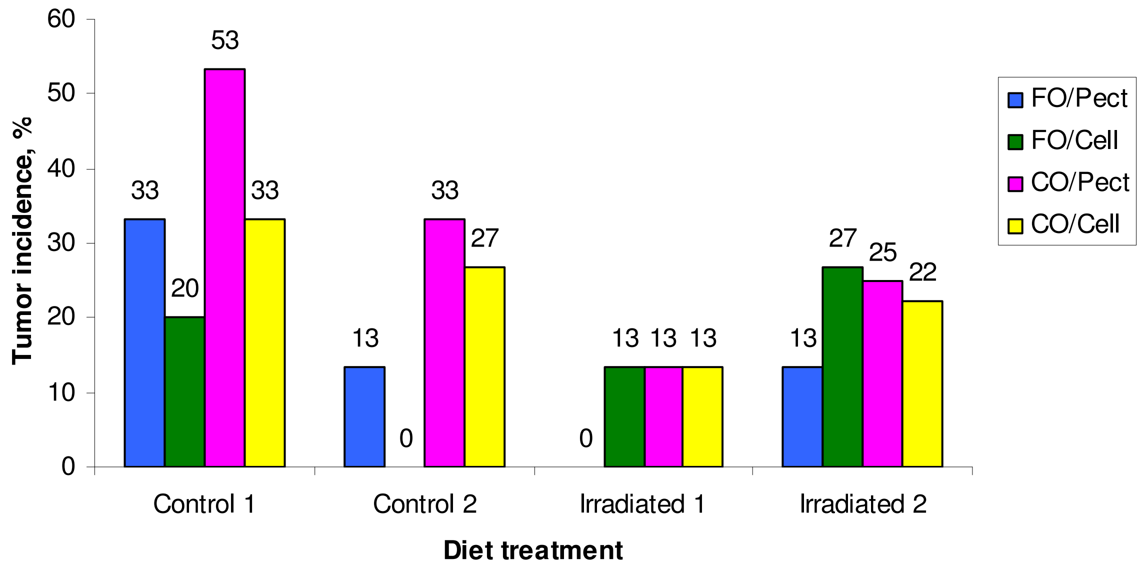


Fig. 21. Malignant colon tumor incidence, all animals included. Diet *(A)* and oil *(B)* treatment group comparisons.
Note: Control 1 group was euthanized 5 weeks later than other three groups of rats.

A



B

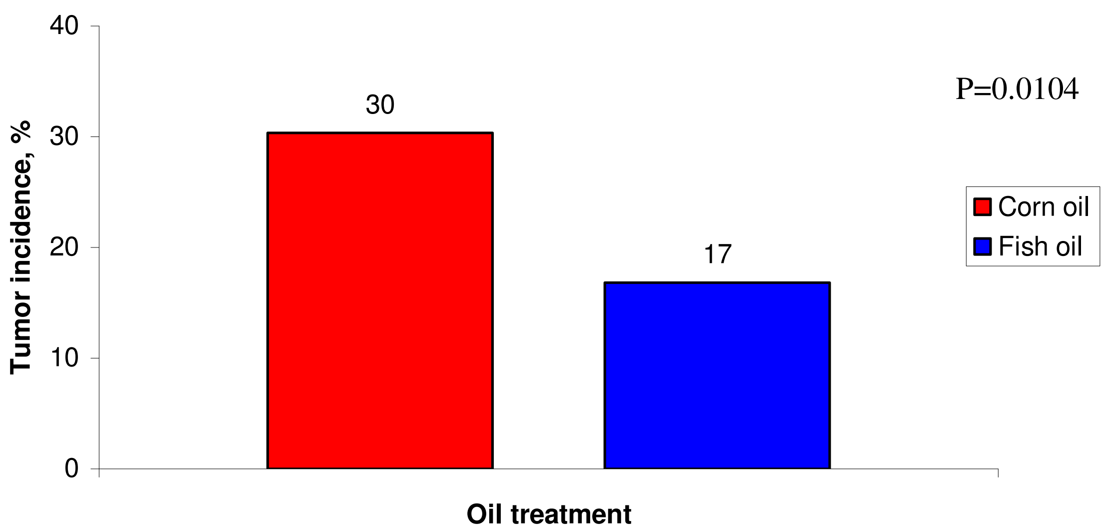


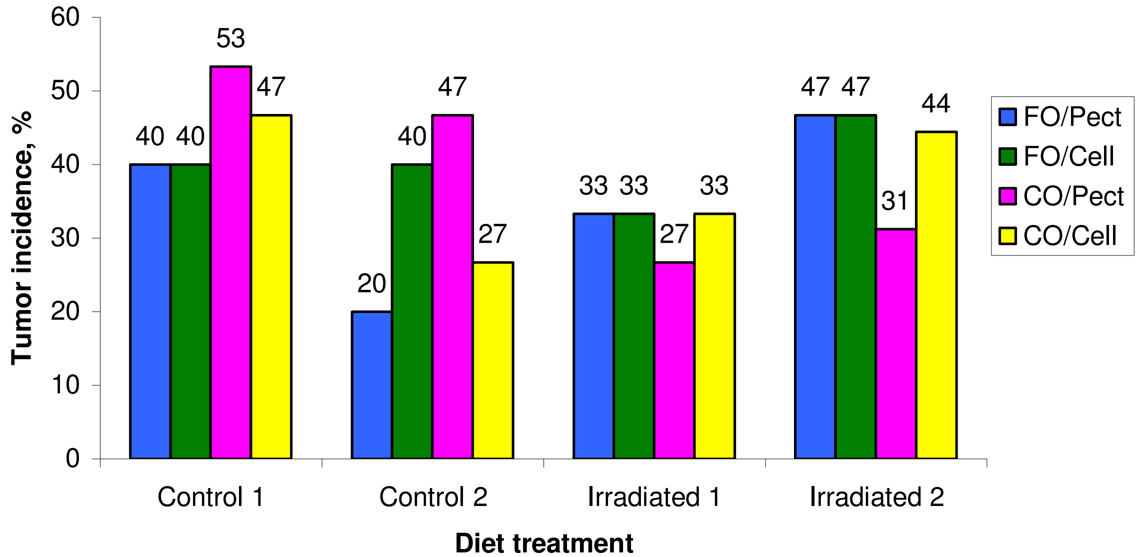
Fig. 22. Malignant colon tumor incidence, only animals survived to the end of study included. Diet *(A)* and oil *(B)* treatment group comparisons.
Note: Control 1 group was euthanized 5 weeks later than other three groups of rat

Though no significant effect of radiation on malignant colon tumor incidence was found, the effect of diet was different for irradiated vs. non-irradiated diet groups (see Figs. 21A and 22A). Thus, for the non-irradiated animals, the fish oil/cellulose diet was found to be more protective against malignant colon tumor incidence when compared to the corn oil/cellulose and corn oil/pectin diets (P=0.0367 and P=0.0038, respectively) and for the irradiated animals fish oil/pectin diet was found to be the most protective when compared to the other diet effects (not statistically significant).

No factors were found to be significant at predicting the probability of having a tumor when all types of tumors at all body sites were considered. However, when all animals used in the study were considered, irradiated corn oil/pectin fed rats appeared to have the lowest tumor incidence when compared to the irradiated rats fed with other diets (Fig. 23A). Conversely, within the non-irradiated groups, corn oil/pectin fed rats had the highest tumor incidence when compared to the rats fed with other diets. When only the rats survived to the end of study were considered, the trend changed in favor of fish oil/pectin diet for irradiated rat groups and stayed almost the same for non-irradiated rat groups (Fig. 23B).

Oil was found to be a significant factor affecting tumor formation in the small intestine (P=0.0375) (Fig. 24B). In contrast to colon tumor results, it was found that rats fed with fish oil-based diets have 2.92 times more chance to develop a small intestine malignant tumor than rats fed with corn oil-based diets, with the highest tumor incidence found at fish oil/cellulose fed animals. However, due to the small number of rats that developed small intestine tumors (21 vs. 218 that had no small intestine tumors), it was not possible to find a correlation between diet and radiation treatment (Fig. 24A).

A



B

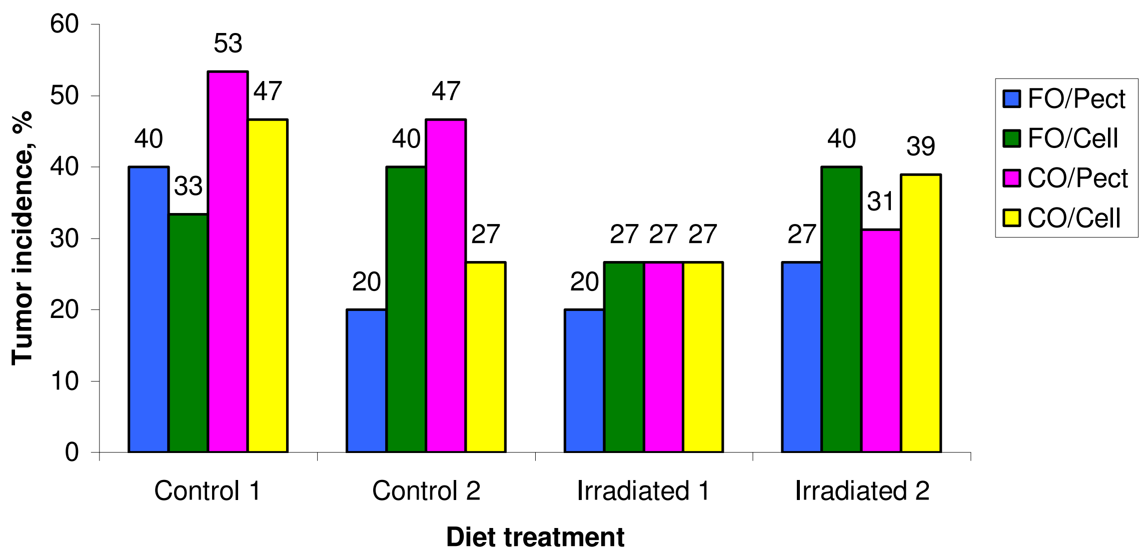
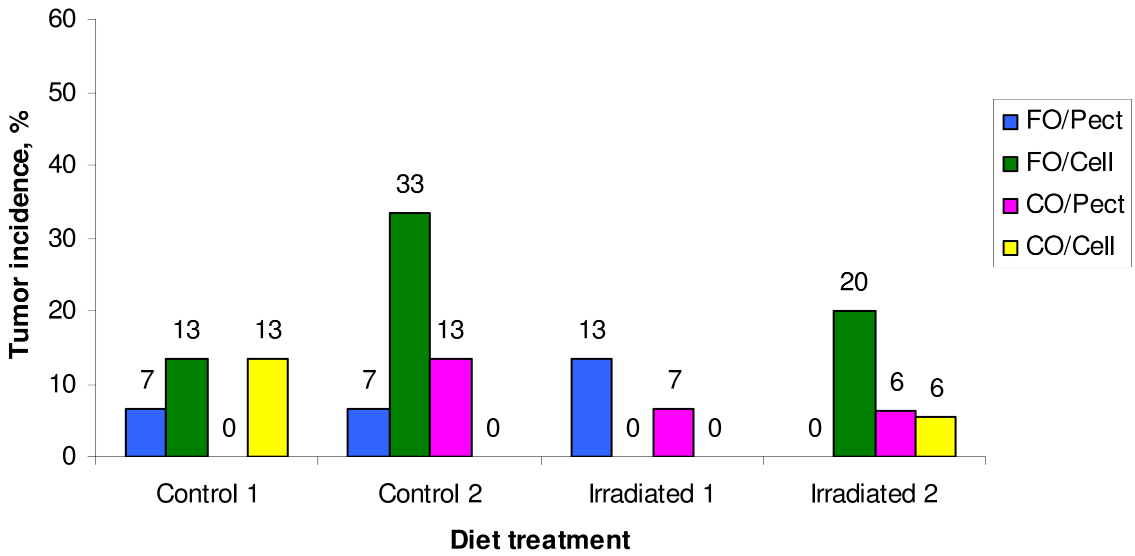


Fig. 23. Whole body tumor incidence (all types of tumors). All animals *(A)* and only animals survived to the end of study *(B)* included. Diet treatment group comparisons.
Note: All types of tumors at any locations are considered. Control 1 group was euthanized 5 weeks later than other three groups of rats.

A



B

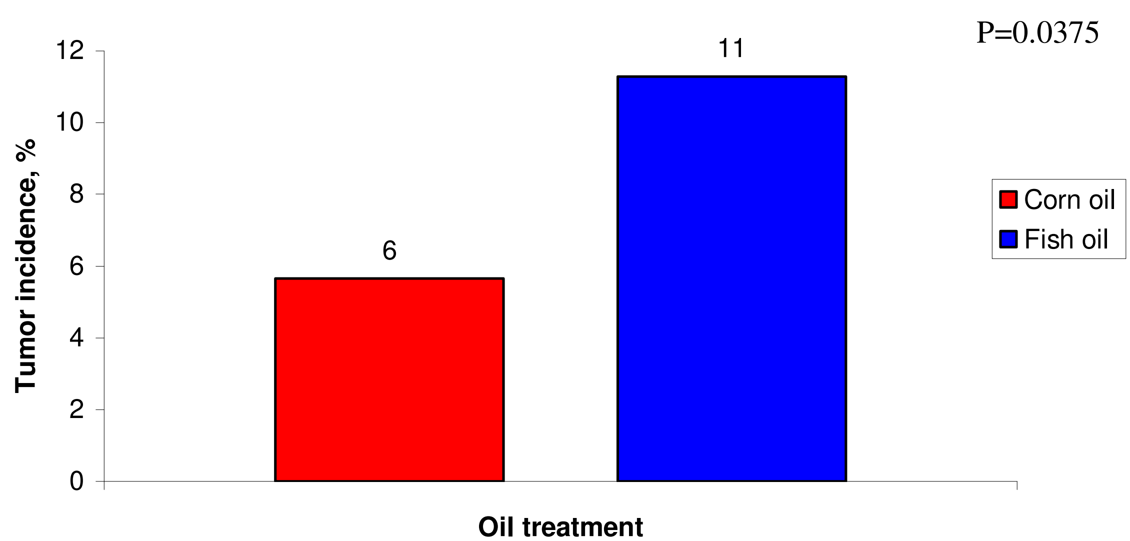


Fig. 24. Small intestine tumor incidence. Diet *(A)* and oil *(B)* treatment group comparisons.
Note: All tumors found in small intestine are malignant. Control 1 group was euthanized 5 weeks later than other three groups of rats.

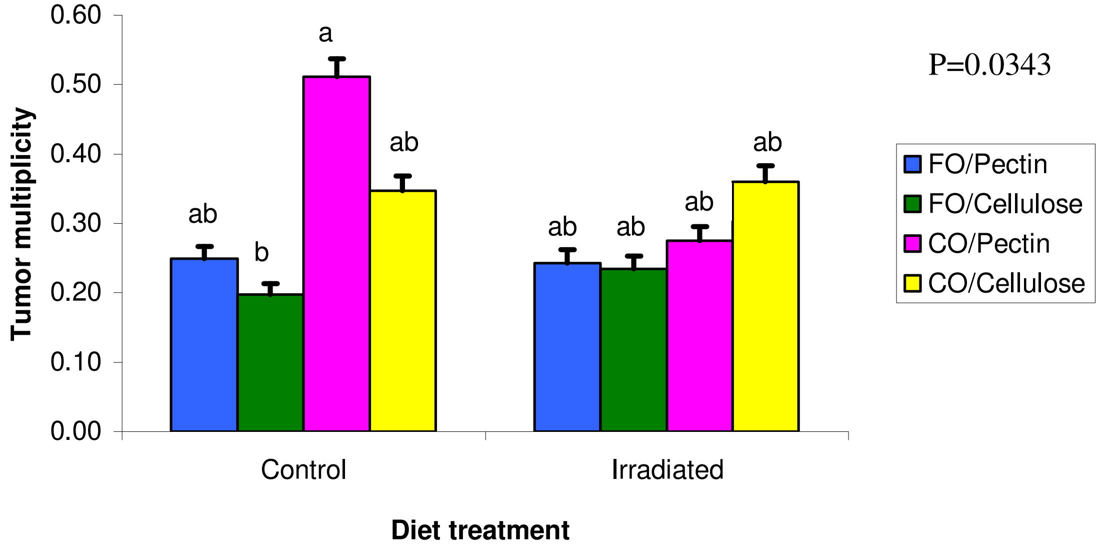
3.4. TUMOR MULTIPLICITY

As in the case of cancer incidence, two separate analyses were conducted: with data from all animals used in the study and with data from the animals euthanized at the main kill-time point.

When all types of colon tumors were considered, corn oil was found to be a significant factor for increasing colon tumor multiplicity (Figs. 25B and 26B). However, when the two radiation treatment groups were considered separately, the effect of oil on tumor multiplicity was significant for non-irradiated groups only (P=0.0419 when all the rats used in the experiment were considered and P=0.0297 when only the rats killed at the main time point were considered). There was no evidence of a significant effect of fiber, diet component combinations or radiation on colon tumor multiplicity. However, there were differences found between irradiated vs. non-irradiated diet groups. Thus, within the irradiated group, the highest tumor multiplicity was observed in corn oil/cellulose fed rats and, in the rats that survived to the end of study, the lowest tumor multiplicity was found in fish oil/pectin fed rats. As for the non-irradiated group, the highest and the lowest tumor multiplicity indices were observed for corn oil/pectin and fish oil/cellulose diet intervention groups, respectively (Figs. 25A and 26A).

The significance of oil effect on colon tumor multiplicity was even more prominent when only malignant colon tumors were considered (Figs. 27B and 28B). Just as in the previous case, the effect of oil on the tumor multiplicity was significant for the non-irradiated groups only (P=0.0152 when all the rats used in the experiment were considered and P=0.0102 when only the rats killed at the end of study were considered).

A



B

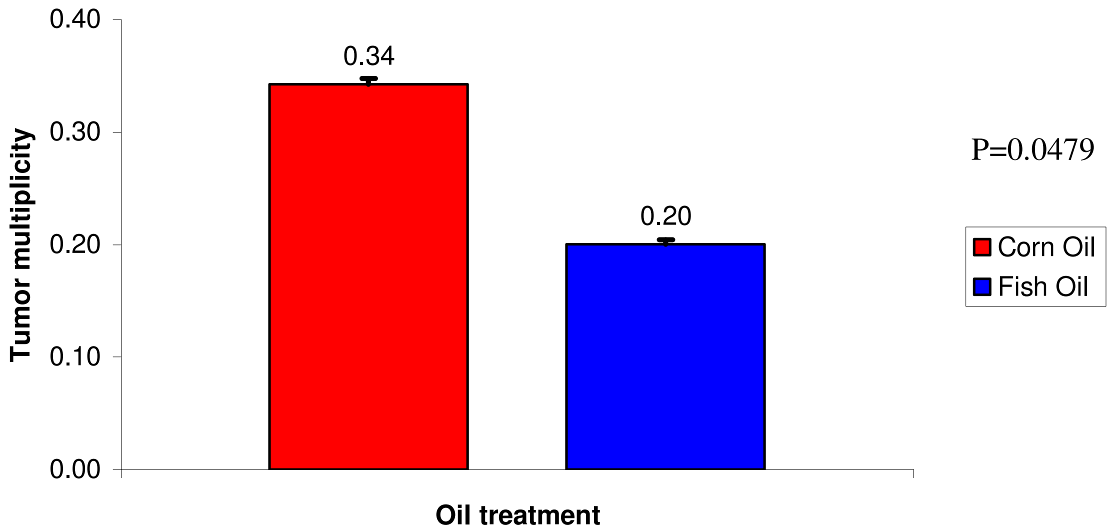
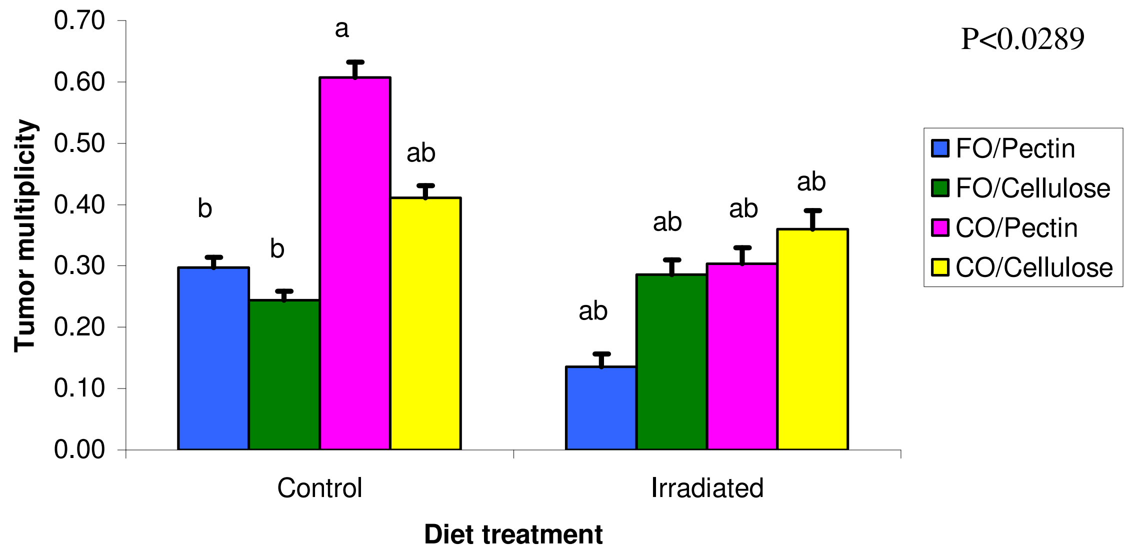


Fig. 25. Colon tumor multiplicity (all types of tumors), all animals included. Diet *(A)* and oil treatment *(B)* group comparisons. Data presented as LS means $\pm$ SE.

A



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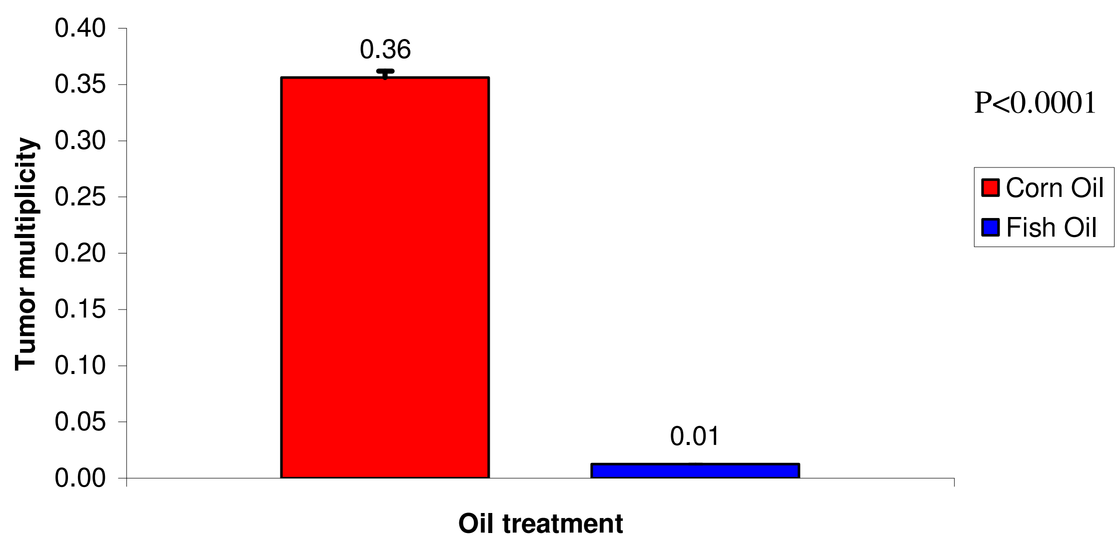
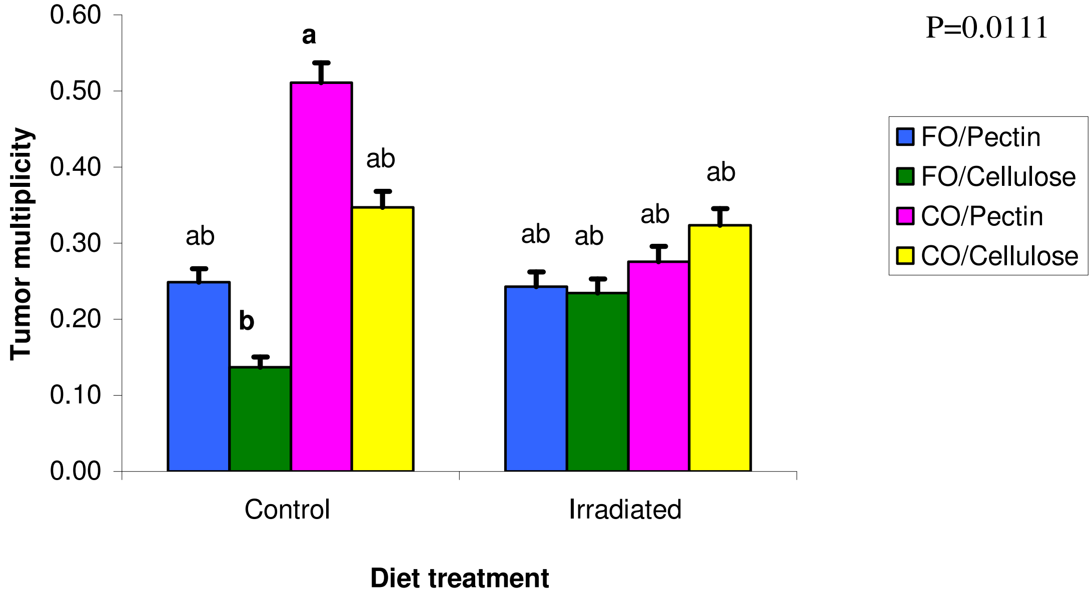


Fig. 26. Colon tumor multiplicity (all types of tumors), only animals survived to the end of study included. Diet *(A)* and oil treatment *(B)* group comparisons. Data presented as LS means ± SE.

A



B

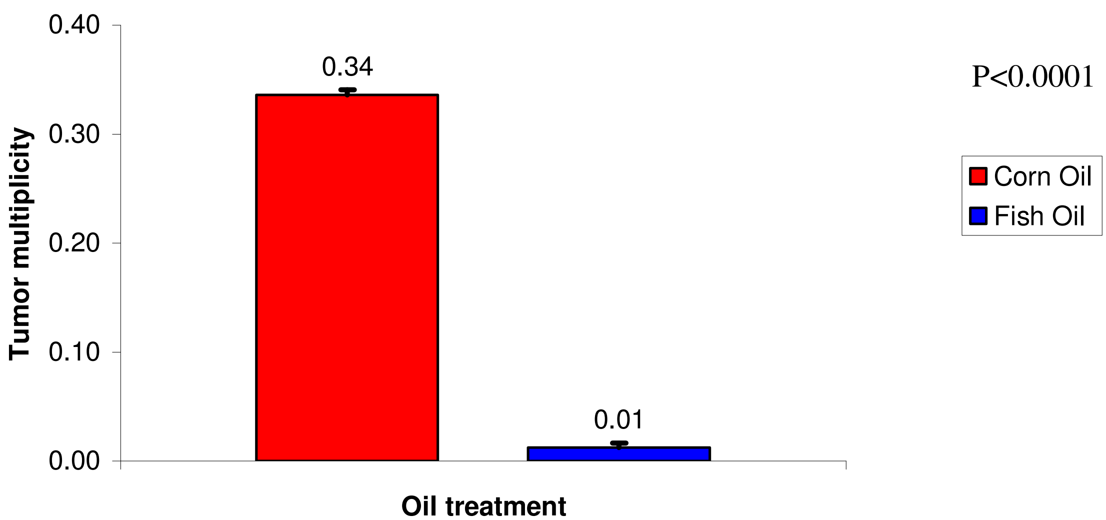
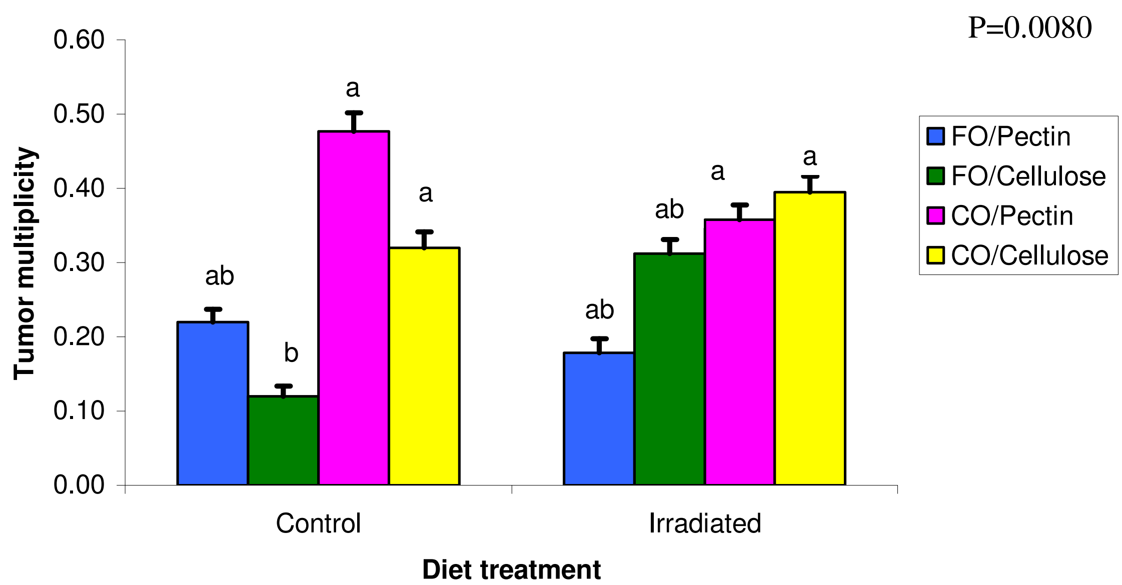


Fig. 27. Colon tumor multiplicity, malignant tumors, all animals included. Diet *(A)* and oil treatment *(B)* group comparisons. Data presented as LS means + SE.

A



B

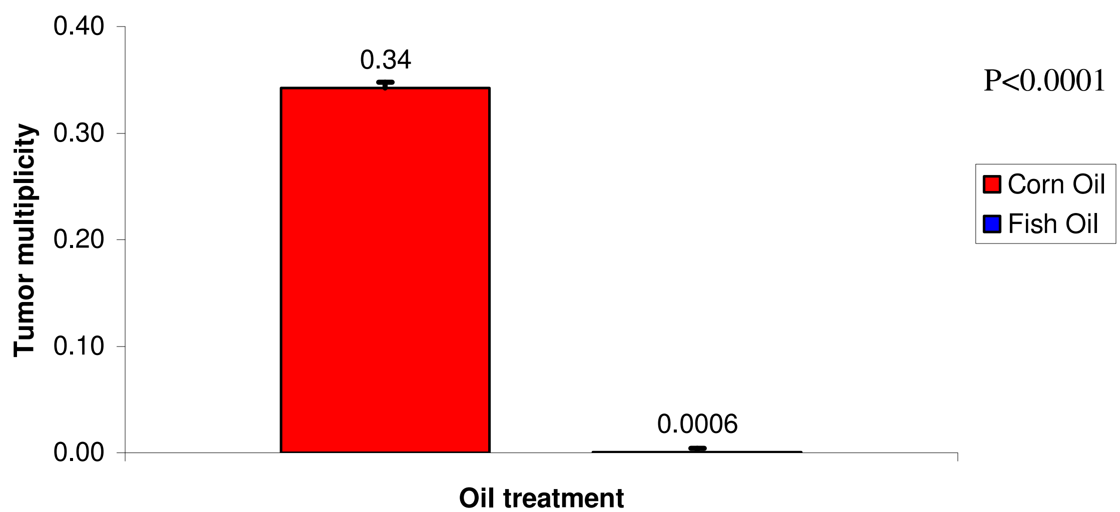
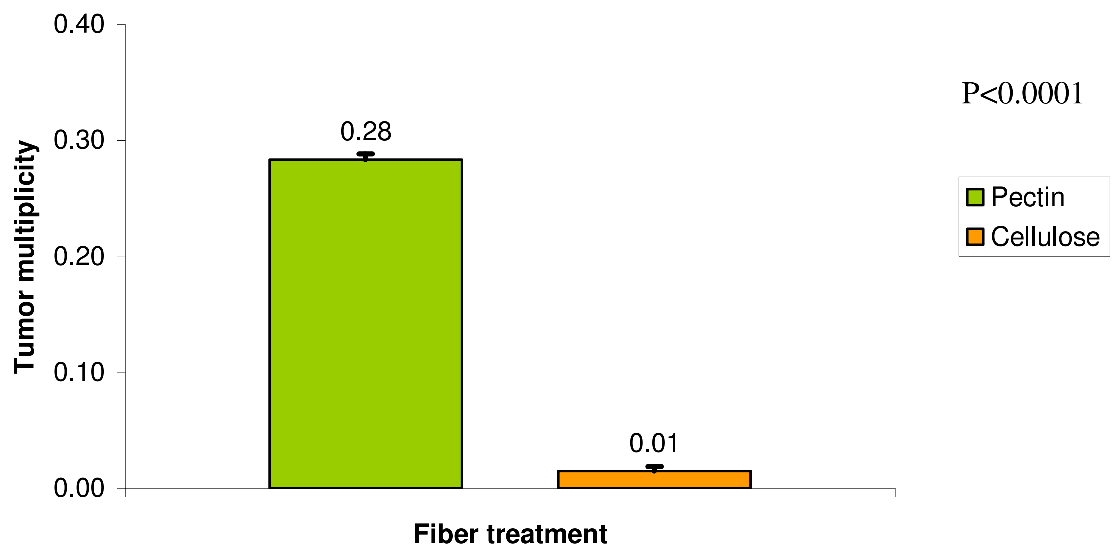


Fig. 28. Colon tumor multiplicity, malignant tumors, only animals survived to the end of study included. Diet *(A)* and oil treatment *(B)* group comparisons. Data presented as LS means $\pm$ SE.

When all the experimental animals were considered, malignant colon tumor analyses have also shown marked difference between fiber groups ($P<0.0001$, Fig 29A), though in overall there was no evidence of significant influence of fiber type on malignant colon tumor multiplicity ($P=0.1209$). However, when only the fraction of rats that survived to the end of the study was considered, no difference in malignant colon tumor multiplicity was found between pectin and cellulose-fed rat groups (Fig. 29B). There was no evidence of significant effect of diet component combinations or radiation on malignant colon tumor multiplicity. However, the effect of diet was different for irradiated vs. non-irradiated diet groups, with the trends analogous to those found in the analysis of all types of colon tumors (Figs. 27A and 28A).

Whole body tumor analyses have not shown any significant influence of radiation, diet components, or their combinations on the whole body tumor multiplicity. However, when radiation treatment was not present, rats from fish oil/pectin diet group had the lowest, and corn oil/pectin the highest, whole body tumor multiplicity compared to rats fed with other diets. When radiation treatment was present, the rats from the corn oil/cellulose groups had higher whole body tumor multiplicity compared to rats fed other diets (Figs. 30A and 30B). Among the irradiated animals that survived to the end of the study, whole body tumor multiplicity was found to be the lowest in the fish oil/pectin diet group.

A



B

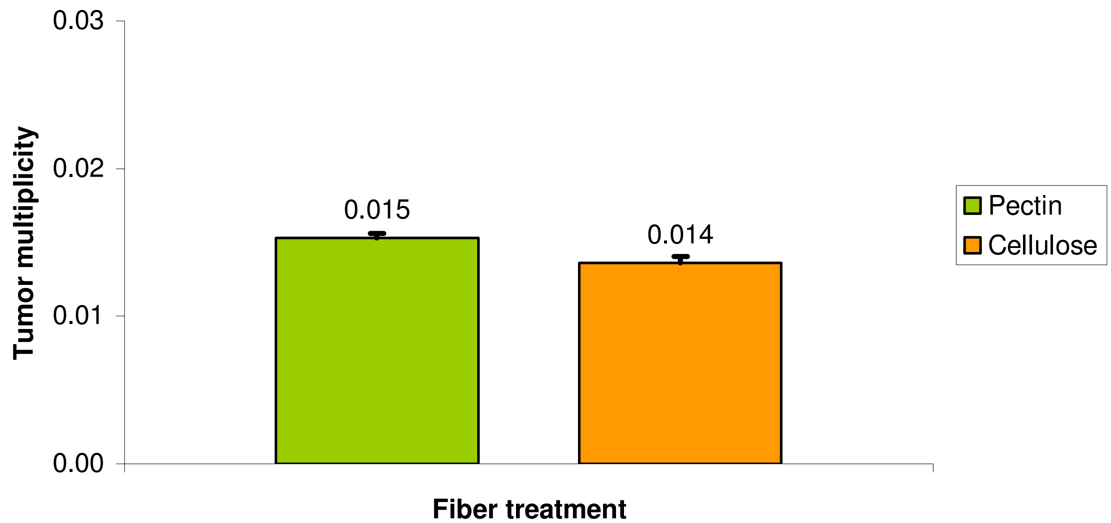
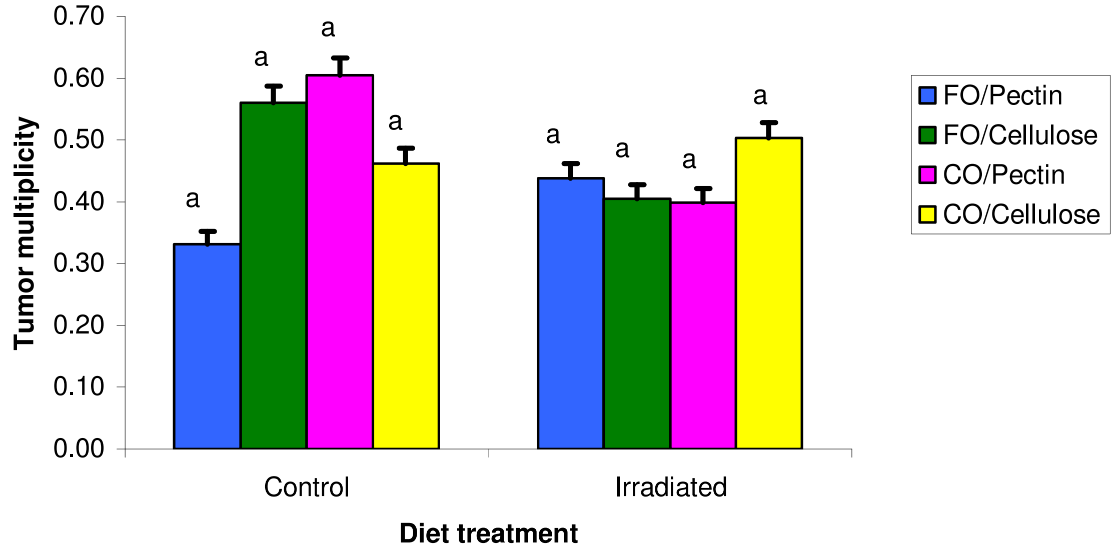


Fig. 29. Colon tumor multiplicity, malignant tumors. Fiber treatment group comparisons. All animals included *(A)* and only animals survived to the end of study included *(B)*. Data presented as LS means ± SE.

A



B

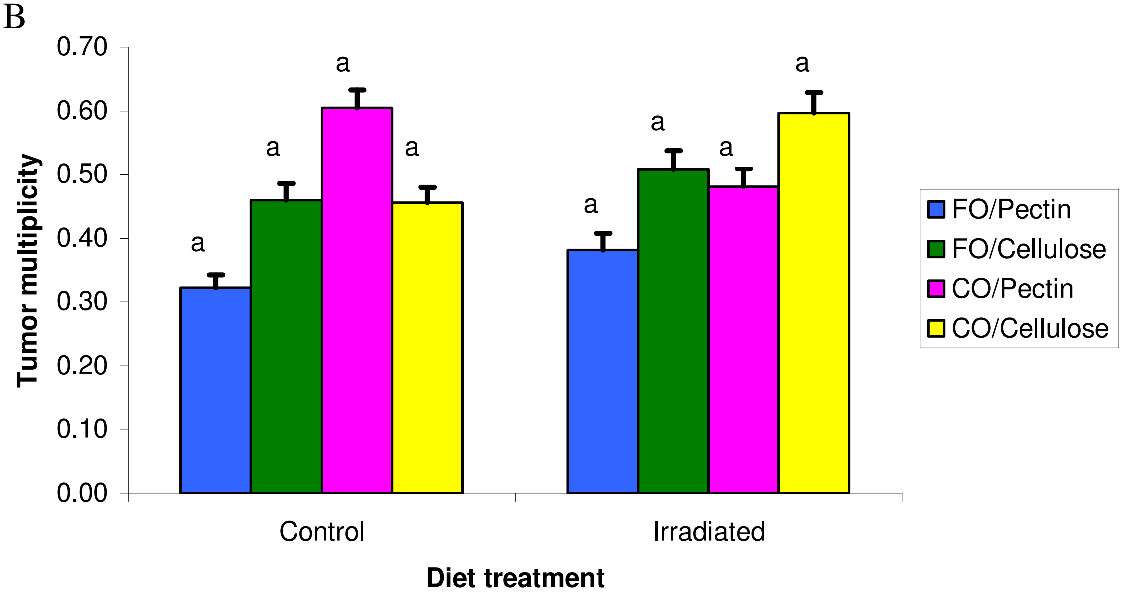


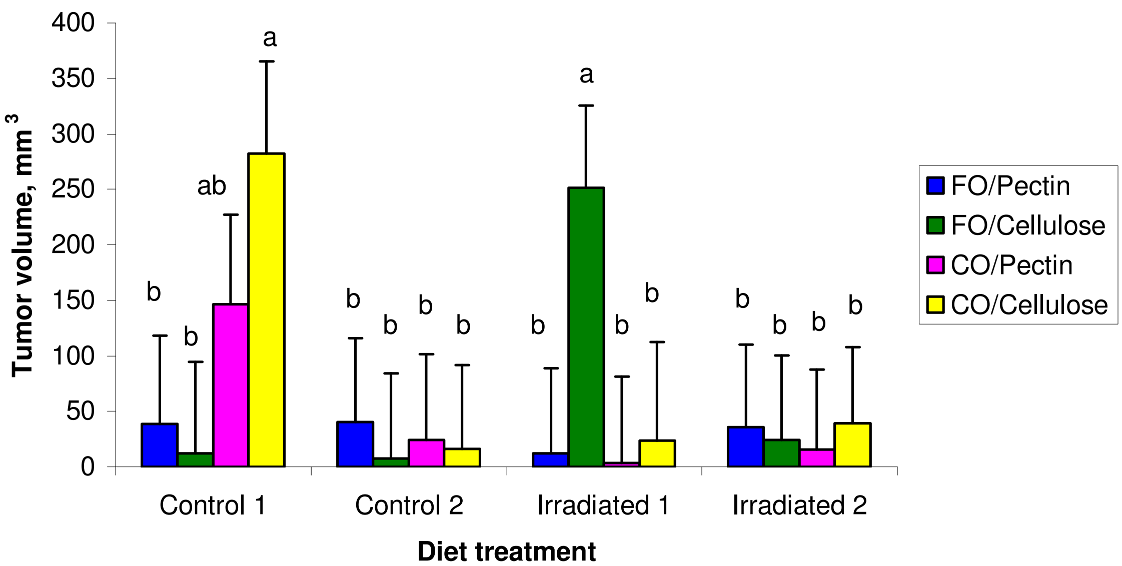
Fig. 30. Whole body tumor multiplicity (all types of tumors). Diet treatment group comparisons. All animals included *(A)* and only animals survived to the end of study included *(B)*. Data presented as LS means ± SE.

3.5. TUMOR VOLUME

The volumes of all types of colon tumors, malignant colon tumors and small intestine colon tumors were analyzed. Due to high data variance within one radiation treatment group, combination of the control and irradiated data *inter se* was not performed.

Analyses of colon tumors of all types showed a marked synergistic effect of oil and radiation on tumor volume (P=0.0408). It was found that tumors of larger sizes are more likely to be found in Group 1 non-irradiated corn oil fed animals and Group 1 irradiated fish oil fed animals (Fig. 31B). Detailed diet group comparison showed significant prevalence of large tumors in corn oil/cellulose fed rats within non-irradiated group 1 and fish oil/cellulose fed rats within irradiated Group 1. However, because of the high variance of the data no similar trends were found in second groups that received the same radiation treatment (Fig. 31A). Concerning similar trends found for the groups that received the same radiation treatment, the non-irradiated rats consuming fish oil/cellulose and fish oil/pectin diets and irradiated rats consuming all other diets except fish oil/cellulose appeared to have colon tumors of smaller sizes. No significant effect of fiber on colon tumor volume was found.

A



B

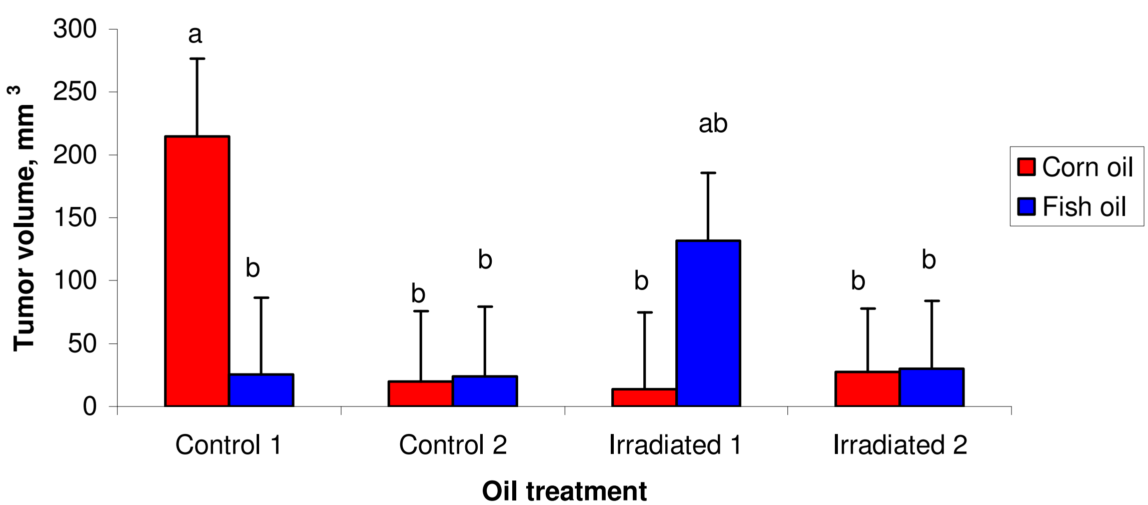


Fig. 31. Colon tumor volume (all types of tumors). Diet *(A)* and oil treatment *(B)* group comparison. Data presented as LS means $\pm$ SE. Tumor volume calculated as a prolate spheroid volume as follows: tumor length*width*height*(π/6).

When only malignant colon tumor volume data were analyzed, a synergistic effect of radiation and diet on tumor volume was found (P=0.0073). For non-irradiated rats, tumors of larger volumes were found among corn oil/cellulose fed rats of non-irradiated Group 1 when compared to the rats fed with fish oil-based diets of the same non-irradiated group, and fish oil/cellulose diet group of non-irradiated Group 2. As for the results of the irradiated-animal data, fish oil/cellulose fed rats of the irradiated Group 1 were found to have the largest malignant colon tumors among all the other diet groups within two irradiated groups (Fig. 32). Concerning similar trends found for the groups received the same radiation treatment, the non-irradiated rats consuming fish oil/cellulose diet appeared to have malignant colon tumors of the smallest sizes when compared to the non-irradiated Group 1 rats fed with corn oil/cellulose diet. No significant effect of fiber on malignant colon tumor volume was found.

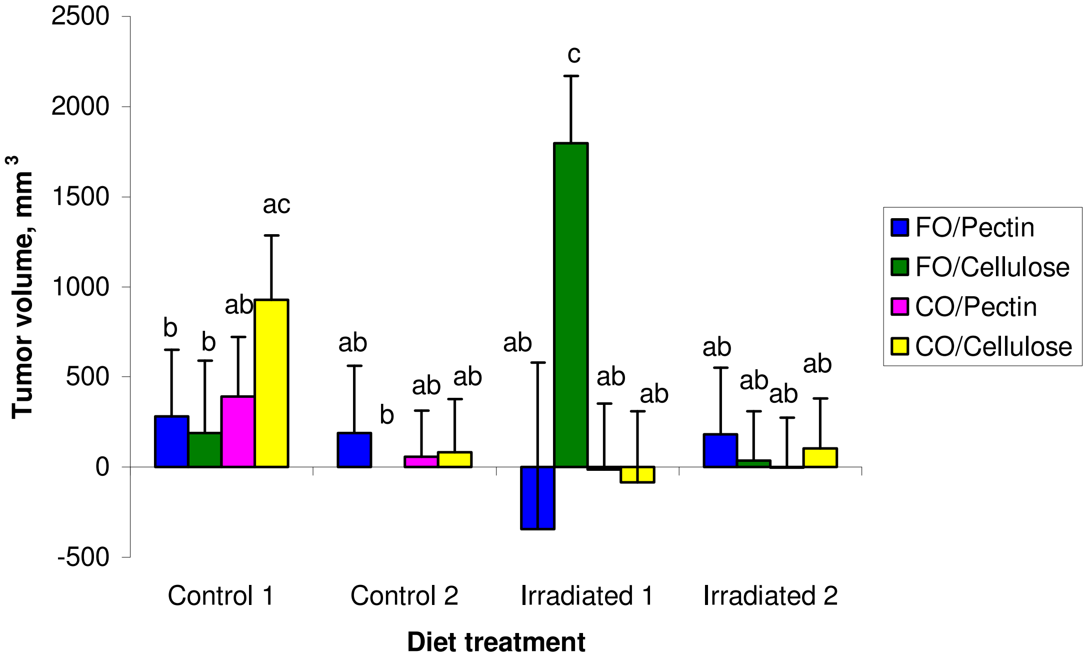


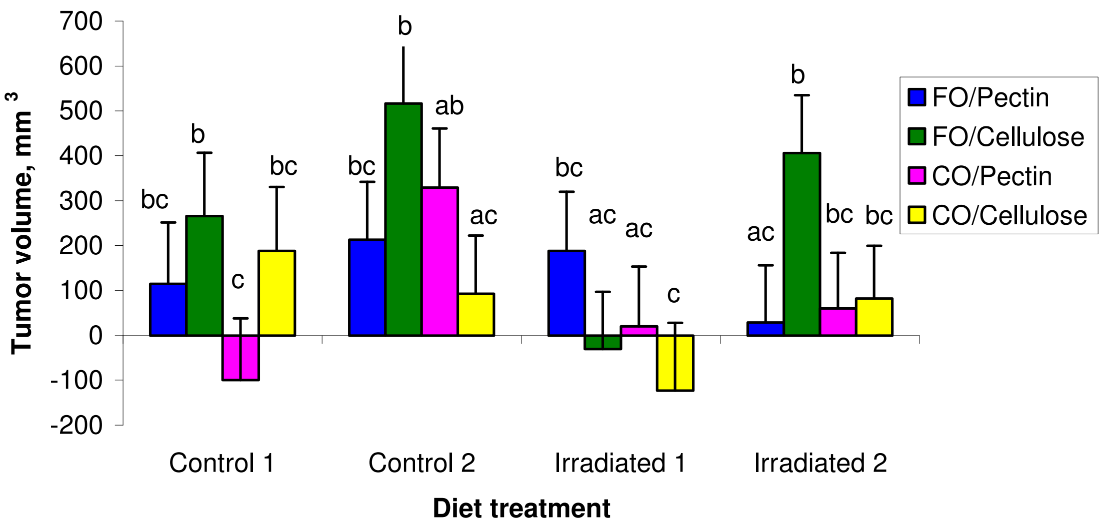
Fig. 32. Malignant colon tumor volume. Diet treatment group comparison. Data presented as LS means ± SE. Tumor volume calculated as a prolate spheroid volume as follows: tumor length*width*height*(π/6).

Due to the low small intestine tumor incidence (21 animals with one small intestine tumor each, out of 245 animals total), four categories of tumor sizes were analyzed: no tumors (tumor volume = 0), small tumors (0<tumor volume<500 mm^3), intermediate size tumors (500<tumor volume<5000 mm^3), and large tumors (tumor volume >5000 mm^3). In this analysis, oil was found to be a significant factor affecting small intestine tumor volume (P=0.0273) (Fig. 33 B). It was found that rats fed with fish oil-based diets had larger tumors than rats fed with corn oil-based diets independent of radiation treatment.

When all the diet groups were considered, among the non-irradiated animals the prevalence of small intestine tumors of larger sizes was found in fish oil/cellulose fed rats when compared to corn oil/pectin fed rats of non-irradiated Group 1 (Fig. 33A). As for the irradiated animals, the fish oil/cellulose fed rats of irradiated Group 2 had significantly larger tumors compared to fish oil/pectin fed rats of the same irradiated Group 2 and fish oil/cellulose, corn oil/pectin and corn oil/cellulose fed rats of the irradiated Group 1. No common significant effect of diet on small intestine tumor size was found within two irradiated groups.

No significant effect of fiber or radiation on small intestine volume was found.

A



B

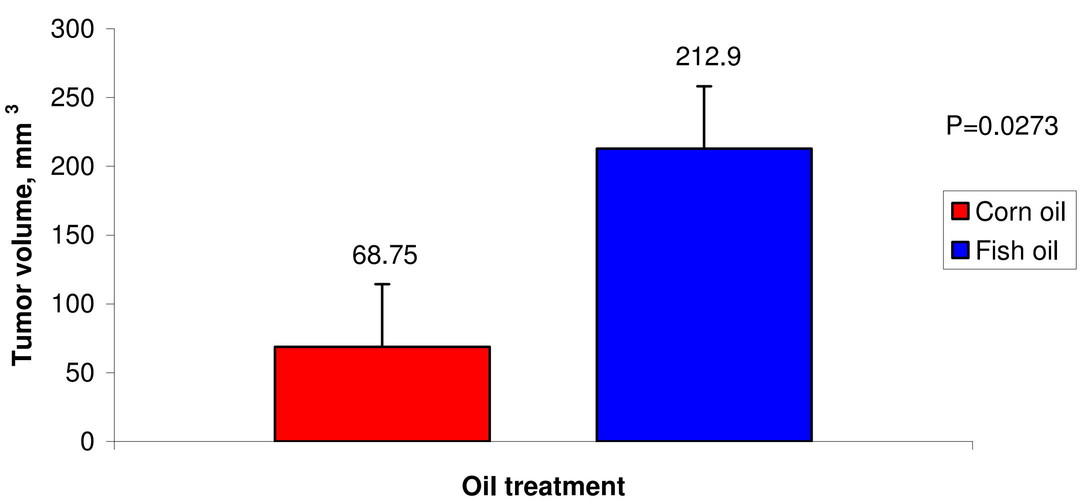


Fig. 33. Small intestine tumor volume, categorized. Diet *(A)* and oil treatment *(B)* group comparisons. Data presented as LS means $\pm$ SE. Tumor volume calculated as a prolate spheroid volume as follows: tumor length*width*height*(π/6).
Note: All tumors found in small intestine are malignant.

CHAPTER IV

DISCUSSION

4.1. FOOD INTAKE AND WEIGHT GAIN

In addition to type of diet, calorie restriction has been shown to be a significant factor positively affecting longevity, delaying and, in some cases, preventing age-associated diseases including varieties of cancers (101-103). Thus, observing the differences in the intake of food by experimental animals of different diet and radiation treatment groups, one would expect to find as a result that the rats with the lowest food intake would have longer lifespan and lower tumor incidence and/or multiplicity. However, data obtained in our study do not support this expectation. Thus, non-irradiated corn oil/pectin fed rats, which consumed markedly less food compared to the fish oil/cellulose and, at the beginning of the study, corn oil/cellulose fed rats, were found to have much higher colon tumor incidence and multiplicity and a tendency to higher whole body tumor incidence and multiplicity when compared to rats from these diet groups. No significantly longer lifespan was observed for this diet group. Similarly, there was no evidence of significantly higher whole body and colon tumor incidence and multiplicity indices for the exposed to HZE radiation fish oil/cellulose fed rats, which were found to have markedly higher food intake at the second control point. In contrast, rats from this diet group had longer lifespan compared to other irradiated rats. Significantly lower food intake of the irradiated animals compared to the non-irradiated ones also did not result in a lower morbidity/mortality rate or increased longevity. Conversely, our data indicate that irradiated rats had significantly lower chances to survive to the end of the study without a tumor (P=0.0005) and that the worsening of physical conditions requiring euthanasia was observed significantly earlier for the irradiated than for the non-irradiated rats. Thus, we may conclude that, even if differences in food consumption and subsequent weight gain had a side effect on physical condition of experimental animals, it was not significant compared to the effects of intervention diets and radiation treatment.

4.2. OILS, FIBERS AND THEIR COMBINATIONS AS COLON CANCER PREVENTING AGENTS

In the current study, fish oil was shown to be more protective against colon cancer than corn oil in non-irradiated AOM treated rats. This is consistent with the results of other studies investigating the effect of fish oil on colon tumorigenesis (61, 62, 64, 65). In rats exposed to Fe-ion radiation the tumor incidence and, especially, the multiplicity, also showed a tendency to lower values when fish oil was present in intervention diets. However, the mechanisms of colon cancer suppression by fish oil may differ depending on presence or absence of radiation.

The possible mechanisms of colon carcinogenesis suppression by fish oil are being extensively investigated. It is known that fish oil downregulates colonic epithelial cell proliferation (77, 78) and upregulates apoptosis (62, 79, 80), thus leading to colon cancer suppression. One of the possible pathways that may explain the protective effect of fish oil against colon cancer is a decrease of cell proliferation through inhibition of prostaglandin (PG) production by n-3 fatty acids (78). 2-series PGs are metabolic products of arachidonic acid (AA, 20:4n-6), essential phospholipid of the cell membranes. They are produced from AA by action of enzymes such as cyclooxygenase (COX). Colonic mucosal prostaglandin E_2 (PGE_2) is considered to be tumor promoter because inhibition of its synthesis has been shown to decrease colon tumor formation (104). Commonly found in fish oil, n-3 fatty acids have been shown to reduce activity of COX-2 in colonocytes (81), decrease AA availability and PGE_2 synthesis (76, 78, 82).

Another major possible pathway of colon cancer suppression by n-3 PUFA is through their effect on RAS gene mutation and expression. It has been shown that RAS activation is associated with increased levels of diacylglycerol (DAG), which in turn decreases levels of protein kinase C (PKC) (86). PKC is known to be involved in the regulation of cell proliferation and apoptosis (87) and its downregulation has been found to suppress the induction of apoptosis (88). Fish oil has been found to decrease RAS expression and mutation (83, 84).

However, cell signaling mechanisms may be altered by ionizing radiation (105) and other protective mechanisms of fish oil action against radiation-induced carcinogenesis may take place. It is known that ionizing radiation induces production of reactive oxygen species (ROS) in cell and subcellular units (23, 24). It is also estimated that primary and secondary ionization events produced at clinically relevant doses (about 2 Gy) of ionizing radaition result in relatively low amount of ROS compared to the amounts of ROS produced during metabolic processes (106). However, even low doses of ionizing radiation were found to have an effect on cytoplasmic signaling (107). Recent studies suggest that although ROS are the initial reagents produced by ionizing radiation, not ROS, but reactive nitrogen species (RNS) are the actual activators of radiation-induced redox-dependent cellular signaling (108). Mechanisms that involve RNS affect cytochrome C and nitric oxide synthases (NOS) balances, resulting in suppressed apoptosis and increased proliferation. As it has already been said, fish oil is known to increase the cytochrome C release to the cytosol as well as decrease inducible NOS (iNOS) expression which is known to stimulate expression of COX-2. Thus, it may shift a balance between colonocyte proliferation and apoptosis in favor of the latter.

Another possible explanation for the suppression of colon carcinogenesis is based not on the action of fish oil alone, but on its interaction with other components present in the intervention diets, resulting in synergistic colon cancer suppression. Thus, it has been shown that fish oil causes changes in intestinal microflora and may result in changes in microflora metabolites, particularly products of fiber fermentation such as short chain fatty acids (SCFA) (109). Chang, et al. (61) in a study that used the same animal/carcinogen model and intervention diets, reported synergistic protective effect of fish oil and pectin against AOM-induced colon cancer in rats. They found significantly lower colon cancer incidence and higher level of apoptosis in fish oil/pectin fed rats when compared to those of the rats fed with other intervention diets. In the present study, for the non-irradiated animals not fish oil/pectin, but fish oil/cellulose combination was found to be protective against colon cancer. It was found to reduce tumor incidence as well as tumor multiplicity. The nature of the discrepancy between the results of these two analogous investigations is unclear. It is possible that it might be caused by differences in diet composition or by other factors not taken into consideration. Omitting this discrepancy, the protective effect of cellulose alone on alkylation-induced colon tumorigenesis is consistent with results of other studies (110-112). However, the exact mechanism of fish oil/cellulose interaction is yet to be determined.

In contrast to this finding, for the fish oil/pectin fed rats exposed to Fe-ion irradiation a trend similar to that found by Chang, et al. (61) was observed. As our data show, not only the irradiated fish oil/pectin fed rats had a tendency to lower colon tumor incidence values, but also colon tumor multiplicity, as compared to the rats fed with other diets. The same trend was observed when all tumors found at all body sites were considered. The question why this combination happened to be the most protective against colon cancer requires consideration of such fiber characteristic as fermentability (56, 57, 72). Fermentability has a significant effect on intestinal microflora, such as anaerobic bacteria. Their metabolic products, short chain fatty acids (SCFAs), forming during fermentation of dietary fiber and resistant starch, were recently found to greatly inhibit colon cancer (73, 74). Butyrate, a product of pectin fermentation, is a highly investigated SCFA and is reported to be a proapoptotic factor in several *in vivo* studies (113, 114). The exact mechanism of interaction of SCFA and n-3 PUFA resulting in suppression of colon cancer development remains to be defined, however, there is strong evidence supporting mitochondria-dependent mechanism. Mitochondria are the major subcellular units responsible for triggering the apoptotic mechanism. It is known that mitochondrial membrane potential (MMP) decreases when mitochondrial cell membranes are damaged by reactive oxygen species (ROS). This allows cytochrome C to translocate to the cytosol, triggering an apoptotic mechanism. Recently Hong, et al.

(115) reported an increased unsaturation index in one of the mitochondrial phospholipids, cardiolipin, in colonocytes isolated from rats fed with fish oil-based diet and then exposed *ex vivo* to butyrate. At the same time they reported decreased MMP, increased cytochrome C translocation to the cytosol and increased caspase-3 activation. It was proposed in this study, that fish oil when compared to the corn oil, increases susceptibility of mitochondrial cell membrane to the damage by ROS due to the increased level of unsaturation of mitochondrial cell membranes. This causes changes in mitochondrial electron transport, which result in decreased MMP. Following release of cytochrome C to the cytosol and activation of caspase-3 result in apoptosis.

It should be mentioned, however, that there is some discrepancy between reports of *in vivo* and *in vitro* investigations regarding butyrate efficacy (75) and it may turn out that the above-described mechanism cannot be approximated to the human *in vivo* studies.

In addition, modulation of cellular signaling by ionizing radiation may have an effect on intestinal microflora. That may explain why the combinations of fish oil and two types of fiber, pectin and cellulose, had effect so different depending on the presence or the absence of radiation treatment. Thus, ionizing radiation has been reported to attenuate secretagogue-stimulated responses as in colon as in small intestine (116, 117), and X-radiation was reported to cause hyporesponsiveness of chloride secretion to secretagogues (118). How these and other factors result on intestinal microflora function is not known to date.

The last index that was measured and analyzed to determine the effect of HZE radiation exposure and intervention diets on colon carcinogenesis is total tumor volume. As our data show, no consistency of this index was observed for the groups of animals that received the same radiation treatment. The fact that tumors of different types can arise in large intestine suggests that tumor volume cannot be considered alone, but the type of the tumor should be taken into account also.

CHAPTER V

CONCLUSION

In this study the effects of Fe ion irradiation and the effects of intervention diets different in lipid (corn oil vs. fish oil) and fiber (pectin vs. cellulose) on AOM induced colon cancer were investigated using rat model.

Radiation was found to inflict significant damage on the health of experimental animals suppressing food consumption ($P<0.0001$) and, consequently, body weight gain ($P<0.05$). The animals exposed to HZE radiation started dying and/or exhibiting pathologies 11 weeks earlier, and at the end of the study have a morbidity/mortality rate 14.2% higher than non-irradiated rats ($P=0.0005$). No significant effect of HZE radiation on colon cancer was found.

The effects of dietary oils and fibers on the health of the experimental animals and on colon cancer development were evaluated. Morbidity/mortality was found to be delayed in rats fed with pectin-based diets when compared to cellulose-based diet regardless of radiation treatment. Similarly, fish oil was found to beneficially affect the health of the experimental animals when compared to corn oil. Morbidity/mortality was found to be delayed in rats fed with fish oil-based diets when compared to corn oil-based diet regardless of radiation treatment. Fish oil was also found to be protective against colon carcinogenesis, significantly reducing both colon tumor incidence and multiplicity in non-irradiated rats ($P<0.05$). It also showed a tendency to reduce tumor incidence and multiplicity values in the irradiated animals. No significant effect of fiber on colon cancer was found.

Finally, diet effects on the general health and colon cancer development was investigated. It was found that rats fed with corn oil/cellulose diet started dying and/or developing pathologies earlier that rats fed with other diets, regardless of radiation treatment. The effect of diet on colon cancer development was found to depend on radiation treatment. Thus in the absence of radiation treatment, fish oil/cellulose was found to significantly reduce tumor incidence and multiplicity when compared to corn oil/pectin diet ($P<0.05$). In the presence of radiation treatment fish oil/pectin was found to decrease the tumor incidence and tumor multiplicity, though not significantly so.

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APPENDIX A

Table A-1 *Mineral mix **AIN-76A** No. 170915 composition*

Component	Chemical formula	g/Kg
Calcium Phosphate, dibasic	$CaHPO_4$	500 .0
Sodium Chloride	$NaCl$	74 .0
Potassium Citrate, monohydrate		220 .0
Potassium Sulfate	K_2SO_4	52 .0
Magnesium Oxide	MgO	24 .0
Maganous Carbonate		3 .5
Ferric Citrate		6 .0
Zinc Carbonate		1 .6
Cupric Carbonate		0 .3
Potassium Iodate	KIO_3	0 .01
Sodium Selenite	Na_2SeO_3-$5H_2O$	0 .01
Chromium Potassium Sulfate	$CrK(SO_4)_2$-$12H_2O$	0 .55
Sucrose, finely powdered		118 .03

Table A-2 *Vitamin mix AIN-76A, No. 40077 composition*

Component	**g/Kg**
Thiamin HCl	0 .1
Riboflavin	0 .1
Pyridoxine HCl	0 .1
Niacin	3 .0
Calcium Pantothenate	1 .1
Folic Acid	0 .0
Biotin	0 .0
Vitamin B_{12} (0.1% trituration in mannitol)	1 .0
Dry Vitamin A Palmitate (500,000 U/g)	0 .1
Dry Vitamin E Acetate (500 U/g)	10 .0
Vitamin D_3 Trituration (400,000 U/g)	0 .0
Menadione Sodium Bisulfite Complex	0 .0
Sucrose, fine powder	981 .0

APPENDIX B

Table B-1 *Mean body weights of dietary intervention groups fed the experimental diets for both non-irradiated and Fe-irradiated rats*

Treatment	Body weight (g) on experimental diets at week ^{a}			
	0 (diet assignment)	3 (Pre-AOM)	29 (20 weeks post 2nd AOM)	At kill
Non-irradiated (groups 1 and 2)				
Fish Oil/ Pectin	**52.1±1.3**^{l,e} **58.3±1.3**$^{l,e'}$	**256.4±4.3**^{l,u,e} 242.3±4.3$^{l,e'}$	495.4±9.6^{s,l,e} **540.4±9.9**$^{l,e'}$	552.4±13.3^{y,s,e} **573.8±12.3**^{e}
Fish Oil/ Cellulose	**52.2±1.3**^{m,d} **58.3±1.3**$^{m,d'}$	**269.9±4.3**^{q,u,m,d} **244.3±4.3**$^{m,d'}$	508.2±9.6^{x,m,q,d} 543.1±9.6^{m,d}	5512.8±13.7^{n,m} **590.2±12.0**^{m,d}
Corn Oil/ Pectin	**52.4±1.3**^{j,c} 58.3±1.3^{j}	**255.5±4.3**^{q,c} 247.9±4.4	**463.2±9.6**^{j,q,s,c} **544.2±9.9**$^{j,c'}$	**517.2±13.3**^{n,s,v} **593.1±12.3**^{v,c}
Corn Oil/ Cellulose	**52.4±1.3**^{i,b} 58.1±1.3$^{i,b'}$	**262.8±4.3**^{i,b} 243.9±4.3$^{i,b'}$	479.0±9.9^{i,x} **532.0±9.6**^{i,b}	**518.4±13.7**^{i,y,b} **582.1±11.9**^{i,b}
Fe- Irradiated (1.2 and 1.0 Gy)				
Fish Oil/ Pectin	**66.6±1.3**^{k,z,w,e} **57.4±1.3**$^{k,e,e'}$	**246.3±4.3**^{k} **219.4±4.3**$^{k,e,e'}$	**476.7±9.6**$^{k,e,e'}$, 529.3±9.6$^{k,e'}$	**501.93±12.6**^{k,e} 553.4±12.4^{k}
Fish Oil/ Cellulose	**65.4±1.4**^{t,d} **57.4±1.3**$^{t,d,d'}$	**244.8±4.3**^{t,d} **229.1±4.3**$^{t,d,d'}$	**463.9±9.9**^{t}, ^{d} 507.6±9.6^{t,f,o}	**520.2±12.7**^{t,d} 564.0±12.9^{t,f}
Corn Oil/ Pectin	**69.4±1.4**^{g,n,w,c} **57.41±1.3**^{g,c}	251.0±4.3^{g} **227.6±4.2**^{g,c}	**475.7±10.3**$^{c'}$ 495.9±9.6$^{o,c,c'}$	**511.3±13.0**^{c} 536.8±11.8^{c}
Corn Oil/ Cellulose	**64.8±1.55**$^{z,h,n,b,b'}$ 57.8±1.4$^{h,b'}$	244.8±4.4^{h,b} **225.7±3.9**$^{h,b,b'}$	**451.2± 11.2**$^{h\ b}$ 484.4± 9.0^{b,h,f}	490.42 ± 14.5 **525.4 ± 11.2**^{f,b}

^{a} Mean ± SE

$^{b-z}$ Means in the same column sharing a common superscript letter are statistically different at P<0.05; means in the same column sharing a common superscript letter and in bold font are significantly statistically different at P<0.0001. Superscript letters in red fond indicate difference between irradiated vs. non-irradiated group; superscript letters in black font indicate difference between diet groups within one radiation treatment group.

Table B-2 *Mean weight gains of dietary intervention groups fed the experimental diets for both non-irradiated and Fe-irradiated rats*

Treatment	Weight (g) gain on experimental diets for periods [a]	
	Intermediate (1st AOM injection – 20 weeks post 2nd AOM injection)	Final (Diet assignment – kill)
Non-irradiated		
Fish Oil/ Pectin	282.9 ± 7.7 [e]	**516.2 ± 9.3** [e]
Fish Oil/Cellulose	284.3 ± 7.6 [d]	522.2 ± 9.2 [d]
Corn Oil/Pectin	266.3 ± 7.7 [c]	507.1 ± 9.3 [c]
Corn Oil/Cellulose	**268.8 ± 7.7** [b]	**503.4 ± 9.3** [b]
Fe- Irradiated		
Fish Oil/ Pectin	239.8 ± 7.7 [f, g, e]	**460.4 ± 9.6** [e]
Fish Oil/Cellulose	252.3 ± 7.5 [g d,]	473.5 ± 9.8 [h, d]
Corn Oil/Pectin	231.9 ± 7.8 [c]	454.1 ± 9.7 [c]
Corn Oil/Cellulose	**218.8 ± 8.0** [f, b]	**444.1 ± 10.3** [h, b]

[a] Mean ± SE

[b-h] Means in the same column sharing a common superscript letter are significantly different at P<0.05; means in the same column sharing a common superscript letter and in bold font are significantly statistically different at P<0.0001 (analysis if variance). Superscript letters in red fond indicate difference between irradiated vs. non-irradiated group; superscript letters in black font indicate difference between diet groups within one radiation treatment group.

Table B-3 *Tumor incidence in dietary intervention groups fed the experimental diets for both non-irradiated and Fe-irradiated rats, all rats included*

Treatment	Tumor incidence [a], %							
	Colon, all types		Colon, malignant		Small intestine		Whole body, all types	
Non-irradiated (groups 1 [b] and 2)								
Fish Oil/ Pectin	33	13	33	13	7	7	40	20
Fish Oil/ Cellulose	27	7	27	0	13	33	40	40
Corn Oil/ Pectin	53	33	53	33	0	13	53	47
Corn Oil/ Cellulose	33	27	33	27	13	0	47	27
Fe- Irradiated (groups 1 and 2)								
Fish Oil/ Pectin	13	13	13	13	13	0	33	47
Fish Oil/ Cellulose	13	33	13	27	0	20	33	47
Corn Oil/ Pectin	13	25	13	25	7	6	27	31
Corn Oil/ Cellulose	20	22	20	22	0	6	33	44

[a] Percentage of rats bearing tumors
[b] First non-irradiated group was killed five weeks earlier than three other groups.

Table B-4 *Tumor incidence in dietary intervention groups fed the experimental diets for both non-irradiated and Fe-irradiated rats, only rats which survived to the end of study are included*

Treatment	Tumor incidence [a], %					
	Colon, all types		Colon, malignant		Whole body, all types	
Non-irradiated (groups 1 [b] and 2)						
Fish Oil/ Pectin	33	13	33	13	40	20
Fish Oil/ Cellulose	20	7	20	0	33	40
Corn Oil/ Pectin	53	33	53	33	53	47
Corn Oil/ Cellulose	33	27	33	27	47	27
Fe- Irradiated (groups 1 and 2)						
Fish Oil/ Pectin	0	13	0	13	20	27
Fish Oil/ Cellulose	13	33	13	27	27	40
Corn Oil/ Pectin	13	25	13	25	27	31
Corn Oil/ Cellulose	13	22	13	22	27	39

[a] Percentage of rats bearing tumors
[b] First non-irradiated group was killed five weeks earlier than three other groups of rats.

VITA

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